Harikesh Bahadur Singh
Birinchi Kumar Sarma
Chetan Keswani *Editors*

Agriculturally Important Microorganisms

Commercialization and Regulatory Requirements in Asia

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Foreword

During the last five decades, application of chemicals in agriculture has helped in managing many pests and diseases, thereby reducing yield losses in crops. However, concerns are often expressed with regard to pesticide residues in food stuff, environmental pollution, imbalance of ecological equilibrium and resurgence of minor pests and pathogens. In sustainable intensification of agriculture through green economy, biopesticides have gained immense significance. Despite some progress in biopesticide production and supply, the scale of its use in India still remains relatively small in comparison to chemical pesticides. Much of the production goes to government agencies for distribution to farmers in integrated pest management (IPM) programmes. The distribution system for biopesticides is underdeveloped in many areas.

This volume on commercial use of agriculturally important microbes in the form of biopesticides includes contributions from vastly experienced Asian experts in a comprehensive manner describing most recent facts and extended case studies. I address the vital issues pertaining to translation of biopesticide research from lab to land. Further, commercialization and regulatory issues concerning biopesticides have also been discussed in a manner that will be invaluable for academicians, scientists, researchers and policymakers.

I congratulate the editors for this useful effort.

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Preface

The past century has witnessed a slow but steady emergence of biopesticides and biofertilizers as potential supplementary and eco-friendly inputs in comparison to their chemical counterparts. Unfortunately, despite considerable research and development efforts, biopesticide use has remained limited to only 2.5 % of the total chemical use in most of the Asian countries. Several technological constraints in these countries have been responsible for the limited adoption which is exemplified by: lack of knowledge, poor availability of standard stable products and situation-specific packages, inconsistent establishment and performance in different crop and agroclimatic domains, inadequate information on safety, and reluctance on the part of big industries for promotion.

The main focus of this book is to review the current status of research, development, and use of these bioinputs in agro-based clusters in developing Asian countries and develop a strategy for addressing critical issues such as policy support, quality control, regulatory management, and public-private participation in implementation of biopesticides in routine agriculture.

The first section will give an overview of the book and will try to develop a consensus on issues of quality requirements, quality control, regulatory management, commercialization, and marketing of agriculturally important microorganisms.

Despite the global progress in establishing a biopesticide supply, the scale of biopesticide use remains relatively small in comparison to chemical pesticides. Thus, the second section will deal with commercialization aspects for implementation of biological control in routine agricultural practices and includes expert views on topics like licensing and enforcing intellectual property rights on hybrid PGPR strains; innovating plant protection strategies in organic farming; identifying and resolving constraints in commercialization of biopesticides; exposing spurious biopesticide trade; and expanding the consortium model in biopesticide research.

Varanasi, India

Harikesh Bahadur Singh
Birinchi Kumar Sarma
Chetan Keswani

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Harikesh Bahadur Singh is head of the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University. Professor Singh has been decorated with several national awards and honours for his key role in popularizing organic farming and translating agriculturally important microorganisms from lab to land. To his credit, he has 20 US patents which he has successfully transferred for commercial production of biopesticides to several industrial houses in India.

Birinchi Kumar Sarma is currently working as associate professor in the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. He received the BOYSCAST fellowship of the Department of Science and Technology, New Delhi, in 2006 for conducting advanced research on agriculturally important microorganisms at the University of California, Davis, USA. He was honoured with the award of 'associate' of the National Academy of Agricultural Sciences, New Delhi, in 2010.

Chetan Keswani is a postdoctoral fellow in the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University. Trained as a biochemist, he has keen interest in intellectual property, regulatory, and commercialization issues in microbiology. He has been a keynote speaker in several national and international conferences to discuss various issues in intellectual property, regulatory and commercialization issues in microbiology.

Part I

Introduction

1 Synthesis of Policy Support, Quality Control, and Regulatory Management of Biopesticides in Sustainable Agriculture

Chetan Keswani, Birinchi Kumar Sarma, and Harikesh Bahadur Singh

Abstract

Growing awareness of organic food production throughout the globe has called for green and sustainable agricultural practices. Agriculturally important microorganisms offer significant benefits in increasing crop yield and improving crop health under both biotic and abiotic stresses. Broad range of microorganisms have been registered and commercialized as biopesticides and biofertilizers all over the world. Analyzing the fact that popularity of biopesticides and their enormous potential are growing, a strong framework for regulation, registration, and quality control for microorganism-based products on global scale is urgently required. Scientists, government regulatory bodies, and industrial representatives must discuss on strategies and future prospects of policy support for ensuring quality of biopesticides in their respective countries.

Keywords

Biopesticides • Policy support • Quality control • Regulatory issues

1.1 Introduction

The Food and Agricultural Organization (FAO) has predicted an increase in the demand of world food production by 70 %. In order to catch up with the supply demand for growing population which is expected to reach ≈ 10 billion by 2050, agro-based economies are employing various strategies for improving crop production (UN 2011). Currently there is an urgent need to enhance current food

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production and livelihood chances from ever-reducing per capita available arable land. In order to meet the food and feed requirements, green revolution has been very impressive, but unfortunately the inputs used in intensive agriculture have led to the several objectionable effects on the human and environmental health. Production of more food from less available arable land is only a phase of challenges; actually the original challenge lies in the fact that crop production should be through safe and sustainable practices.

The global biopesticide market is estimated to reach US$ 6.6 billion by 2020 with expected growth at CAGR of 18.8 % from 2015 to 2020. In India, biopesticide market is forecasted to show high growth with projected compounded annual rate of 19 % over the 2015–2020 period (https://www.kenresearch.com/agriculture-and-animal-care/crop-protection/india-biopesticides-market-research-report/669-104.html). Asia-Pacific region represented largest consumption and demand for bioinsecticides and shared 27.7 % and 38 % of volume and value, respectively, of the global market in 2013. Biopesticide market in this region is projected to attain fastest growth from 2015 to 2020 with CAGR of 17.8 % (http://www.mordorintelligence.com/industry-reports/asia-pacific-biopesticides-market-industry). Consumption of pesticides in countries like India, China, and other developing countries of the region is booming in order to fulfill the demand of the growing population. However, reduced availability of arable land per person in India, China, and other countries in this region is a matter of concern especially in Southeast Asia where per person availability of arable land is decreasing by 0.1 ha per person. Increasing demand for pesticide-free food and organic products is another key driver in the biopesticide market growth that encourages sustainable farming practices. The market of biopesticide however is mostly constrained by the farmer's poor awareness on application of microbial agents and availability of the products. Additionally, low shelf life of formulated products forces farmers to frequent the application of biopesticides, thus resulting in increased costs.

1.2 Quality Assurance

Quality assurance (QA) of biopesticide refers to the protection of a preferred level of quality parameters in formulated product, particularly by careful inspection of every stage of the production and delivery. As per OECD, product quality must be ensured through quality assurance program coordinated by expert personnels.

1.2.1 Issues Related to the QA of Biopesticides

Most of the biopesticide products undergo various abiotic stresses during production, and application stages resulted in their inconsistence field performance and poor shelf life. Likewise, the microorganisms released in the fields through formulated product must be acclimatized to local environment. All these performance issues depend upon the selection of superior strains of bioagents. Thus, the purity

and viability of their culture must be protected and ensured irrespective of laboratory practices to minimize the farmer's risk. Guarantee on the performance ability of parent culture in any given environment must be assured for consumer confidence in the product.

Microbial consortium-based products also possess multiple benefits (Jain et al. 2013). QA for such microbial consortium product needs careful inspection in terms of cultural methods, mass production, and microbial composition in the product.

Addition of various survival factors for suppression of microbe's metabolism is an important aspect for improving shelf life of the formulated products. Information regarding the microbe survival and multiplication in favorable environments must be mentioned. A quality product with maximum satisfaction of the consumers has to be the watchword of any production system. Quality must, therefore, be ensured at all costs (Parmar 2010).

1.3 Standard for Registration of Microbial Biopesticides

To promote registration of biopesticides, simplified registration procedures and acceptance of generic registration data for new products containing strains already registered under national law would be helpful in accelerating the registration process (Kulshrestha 2004). Manufacturers can register their products as temporary regular products. This system allows commercial producers of those microbial pesticides evaluated as generally safe to obtain provisional registration and continue to develop a market, while the product is undergoing full registration; this reduces commercial barriers to product development. The data requirement for registration in temporary section is less stringent than for regular section. For example, efficacy data on specified crops should be required from two locations over two seasons for temporary registration, while the same should be required from three locations for permanent registration. Data on product characterization, efficacy, safety, toxicology, and labeling must be submitted while applying for registration. The established quality standards must be met, with reference to content, virulence of the organism in terms of LC_{50}, moisture content, shelf life, and secondary nonpathogenic microbial load. Protocols for assessing these quality parameters have been prescribed (Rabindra 2005). A long-standing issue is the poor quality and unreliability of some products, which has had a negative impact on farmer confidence and, as a result, farmer demand (Kennedy et al. 1999). Survey to test the quality of biopesticides is conducted, and while some manufacturers clearly meet accepted standards, other reports indicate quality concerns, especially from new and inexperienced producers (Ignacimuthu et al. 2001). A system of referral laboratories accredited by the national agencies for quality testing has to be established to quality standards.

Indian standard includes six parameters on which information is required for registration of microbial biopesticides. This includes biological and chemical characteristics of formulation, bioefficacy, toxicity, packaging, and labeling.

Biological and chemical characterization includes information about systematic name (genus and species), strain name, and common name of microorganism, if

any. It also requires information regarding the source of origin, habitat and morphological description of microorganism used in the formulation, test methods, qualitative analysis, and shelf-life claims.

Chemical composition of the formulated product, cfu/g of the product, percent content of the biocontrol organism in the formulation, and the nature of biomass are required. Physical characteristics such as percentage of carrier/filler, wetting/dispending agent, stabilizers/emulsifiers, contaminants/impurities, and moisture content of the product are also required. Information related to the process of manufacturing and production including type of fermentation, biological end products, and methods of mass multiplication is necessary for registration.

1.4 Regulatory Barrier for Commercialization of Biopesticides

The registration and commercialization of biopesticides is monitored by regulatory framework that was intended for the regulation of synthetic (chemical) pesticides. Hence, the system has a number of features that did not make it amenable for the registration of biopesticides, and a number of adjustments have to be made to facilitate their registration. Biopesticides include wide range of living entities and nonliving substances that differ distinctly in their properties, like composition, physical state, mode of action, and so on. The government policy also requires that the efficacy of a biopesticide product must be ensured and quantified in order to support the claims. Only authorized biopesticide products can be marketed legally for crop protection.

The Organisation for Economic Co-operation and Development (OECD) guidelines only authorize the biopesticides with minimal or zero risk. According to the OECD guidelines, microbial biopesticides, microorganism, and its metabolites should not pose any threat of pathogenicity and toxicity to other nontarget organisms when exposed to the product; the microorganism does not produce any genotoxin; carrier and additive substances used in the formulated product must be of low toxicity (OECD 2003). The data portfolio required for biopesticide registration is generally a modified form of the data portfolio used by authority to risk assessment for chemical pesticides. Data includes information regarding toxicity evaluation, mode of action, host range, etc. However, performing trails for these information is expensive for producers, and it is discouraging for commercializing biopesticides. Therefore, a proper system for biopesticide registration ensuring their safety and consistency which does not inhibit commercialization is urgently needed. Until very recently, it can be said that governmental agencies on most of the countries' regulations except in the USA were unfamiliar to biological pest management and are slow to realizing the benefits of new suitable regulatory process for biopesticides rather than regulating them via same process as chemical pesticides.

The decision related to authorization of a biopesticide product is made by an expert panel formed by regulatory authority. In many cases regulators lack expertise in biopesticides, resulting in delay in decision, and may further request the applicant

to even provide them more data. It is also possible that regulating authority using the registration model of chemical pesticide requests inappropriate information. Some regulatory authorities in the UK, for example, have acknowledged that basing the regulatory system for biopesticides on a chemical pesticide model has been a barrier to biopesticide commercialization (ACP 2004). A key question is whether the regulator, having recognized a problem, is able to do something about it. Social science theory indicates that government regulators and other bureaucratic organizations are vulnerable to "goal displacement," during which they turn their focus away from achieving outcomes and instead concentrate more on internal processes. This can lead to systemic problems and stand in the way of introducing innovations into the regulatory system. This is not to say that regulatory innovation is not possible, and where there is sound evidence that a particular group of biopesticides presents minimal risk, the regulators have modified the data requirements. For example, the OECD regards semiochemicals used for arthropod control as presenting minimal hazard, with straight chain lepidopteran pheromones that form the majority of semiochemical-based biopesticides being thought sufficiently safe as to justify "substantial reductions in health and environmental data requirements" (OECD 2001).

1.5 Need for Commercialization of Biopesticides

Most of the research in the field of agriculture in Asia has been mainly fundamental. Green revolution has resulted in onset of applied and advance research in various agricultural disciplines and other applied areas where scientists realized the importance of effective research to reach to the farmers and the ease with which it could enhance the economic status of the farmers. In Indian subcontinent, fertilization and plant disease management practices were mostly conventional such as the use of cow dung, leftovers of the previous crops, and rouging and burning of diseased plants. But it was only after the emergence of chemical fungicides on global commercial scale that the production of crops escalated and changed the agricultural practice scenario forever. Now same is the situation with natural antagonistic organisms. The scientists from all over the world have screened, selected, and tried the most effective isolates against wide range of plant pathogens.

In this sequence the next rational step is to commercialize these biocontrol products. Commercialization of these eco-friendly biocontrol agents is necessary to lower the harmful effects caused by injudicious used of chemical pesticides. In many cases developing countries are continuously applying various harmful chemicals which have been banned in other developed countries. In such scenario it is well anticipated that development, production, and successful commercialization of biocontrol agents will effectively reduce and eliminate the use of hazardous chemicals and ultimately reduce their deteriorating impact on human and environment health. In developing countries, it is very tough to convince the large-scale farmers to incorporate biocontrol products in their disease management practices as their illiteracy and overdependency on chemical pesticides is a generation-old practice.

In India where major portion are farmers, it is roughly estimated that only 1.5–2.0 % of plant protection market is occupied by biocontrol products (Annon 2011).

It has been assumed that research related to the biocontrol is very fascinating and successful at the initial scale and showed very good results in vivo but failed to replicate its nature in large-scale or infield condition. Additionally there are very few products which have been commercialized. Globally, about 80 biocontrol products have been commercialized. These products are constrained in their application as they can manage limited number of plant pathogens and have not been tested on various major crops before being commercialized. Furthermore, relatively very little investment has been made in the production of commercial formulation of biocontrol agents probably due to the cost of production, testing, registration, and marketing (Singh et al. 2002, 2004). The various efforts made in commercialization of biocontrol agents may be classified in three groups based on level of difficulty and potential of repeat sales. The first group includes direct application of the biocontrol agents precisely on the infection area when and where needed. This process is carried to prevent the pathogen movement by applying large inoculums of biocontrol agents as in case of seed treatment with antagonistic microorganism for protection of seeds against soilborne pathogens. Biocontrol agents commercialized to date mostly come under this group except *Gliocladium virens* GL-21 which is applied only in close vicinity of seed or plant roots in order to facilitate rapid colonization.

Plant growth-promoting rhizobacteria (PGPR) constitutes the second category and is applied at one place, e.g., on seed surface with the viewpoint that they will colonize the plant roots and protect them from various plant pathogens. This group exemplifies the strategy of augmentative application where the antagonists persist with plant throughout the life of host and rapidly increase the population in the rhizosphere (Cook 1993). This category presents a more challenging approach to biocontrol as the bioagents are subjected to greater competition under environmental conditions.

The third class belongs to the biocontrol agents, which are applied on the infection area once or several times. Most of the successful examples of this category are biocontrol agents of insects and perennial weeds. The reason behind the success of this group of biocontrol agents is that they persist on the host for a longer period for their survival and multiplication. However, despite many success stories of effective control, their commercialization have been relatively slow, and therefore application is mostly restricted to greenhouses.

Major reasons for failure of the biocontrol and problems associated with their commercialization are as follows:

1. Low disease pressure for an effective test.
2. The treatment favors the increased damage from nontarget diseases.
3. Colonization of the roots affected by the introduced strain or the loss of ecological competence by strain is variable.

4. The production of antibiotics, wherever it is necessary for the effective action of antagonist, is either too low in quantity to be effective for controlling the disease (Cook and Baker 1983).

Nevertheless, these problems can be managed by selecting effective strains and improving their efficacy through modern biotechnological interventions. Other limitations related to commercialization are institutional, technical, and unrealistic expectations from these biocontrol products. However, overcoming these barriers is necessary to successfully develop commercial biocontrol products.

The efficacy of formulated biocontrol product to compete in rhizosphere varies with changing soil conditions. After isolating an effective biocontrol agent with high rhizospheric competence, the further research must be directed toward the large-scale production of the particular strain. The multilocation field trials under different soil on different crops against various pathogenic fungi are necessary requirements for the developments of the product. Various biocontrol fungi along with disease suppression also offer biofertilizer activity and are vital in ameliorating of abiotic stresses.

Selection of substrate which is suitable for formulation is another challenge. Wide ranges of substrates have been used for commercial production of biocontrol formulations. Agricultural wastes would be economically viable, while other organic various substrates have been developed for economical mass multiplication of biocontrol fungi for field application such as diatomaceous earth granules, molasses, sorghum seeds, powdered ryegrass seeds, wheat bran, sawdust, sugarcane and maize straw, molasses-yeast medium, tapioca rind or thippi substrate, alginate-wheat bran, coconut coir pith, vermiculite-wheat bran, banana pseudo-stem pineapple peeling compost, vermicompost, etc. (Keswani 2015).

1.6 Constraints in the Production of Microorganism-Based Biopesticides

Poor shelf life of formulated product, inconsistent performance in natural conditions, possibilities of contamination human pathogens, lack of application technology, small market size, and low investment are various factors limiting the production of biopesticides. Interest in using biopesticides by farmers can only be enhanced by production of superior formulation, because various low-class products will jeopardize the whole reputation of biopesticides. Biopesticide development and production is a long process which includes selection of superior strain, screening, mass production, selection of suitable carrier and other inert for formulation, assessment of the shelf life, and efficacy of developed formulation. Several reports indicated that the product with low cfu of biocontrol agents and contaminated with other microorganisms has been sold in the market (Singleton et al. 1996; Alam 2000; Arora et al. 2010). Due to low cfu count, it is obvious that their performance in field would be inconsistent. Shelf life of products depends on the several factors such as production procedure, carrier substances, packaging, transport, and storage. A

large-scale production technology is required for biocontrol agents that do not produce enough spores in liquid media. Unfortunately mass production technologies based on solid substrates are not commonly available (Connick et al. 1990). Selection of the cost-effective technology for the mass production viable and propagules is a matter of concern. One of the major objectives in biopesticide production is to maintain the viability of the active ingredient possibly for 2 years. The main challenge faced by producers is maintaining the shelf life of microorganism during the storage period. If the product carries less numbers of active ingredients due to shorter shelf life, the overall performance of the formulation will be affected.

1.7 Future Prospects

To improve the global market perception of biopesticides as effective products, the industry should establish a certification process to ensure efficacy, quality, and consistency of the biocontrol products. The data should be in the public domain and should be easily available to the farmers and extension personnel. In addition, various questions must be addressed before harping on the success of biocontrol in comparison to the chemical control methods, viz.:

(a) What is the method of introduction of pathogen and its spread?
(b) What is the relationship between the population density and damage?
(c) What are the effects of various environmental conditions on the efficacy of introduced biocontrol agents?

We have to find a way out of this quagmire to assume that our commercialized biocontrol products have the effectiveness and safety as their chief traits. Only then commercialization of biocontrol products can be assumed to be successful. For biocontrol to be more acceptable, the concept that the disease should be managed rather than completely controlled is useful and has to be instilled in the mind of end users. Besides, the market size, inconsistency, methods of production, formulation, and distribution have made commercial companies reluctant to support the sustained efforts in biocontrol research. Recent advances in genomics, transcriptomics, proteomics, and metabolomics can guide to the development of next-generation production with increased shelf life.

1.8 Conclusion

To shape the future of biocontrol of plant diseases at a global level, the big challenge is how the modern technology can be translated from lab to land. Although, after so many years of intensive research and despite all the success stories of biocontrol, it is evident that the number of commercially available biocontrol products is far lower than chemical counterparts. It can be stated that the commercialization of biocontrol products is far behind due to the inappropriate policies and tedious

registration process worldwide. Thus, it is vital for the policy maker to facilitate the registration process and at the same time be stringent enough to regulate the spurious products in the market. Moreover, biocontrol research will only be blue-sky research if trust and awareness among the farmers are lacking. Thus, government, industries, and academia should join hands in sensitizing the farmers to adopt eco-friendly and sustainable farming practice and agri-inputs.

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Part II

Issues in Commercialization of Agriculturally Important Microorganisms

Superior Polymeric Formulations and Emerging Innovative Products of Bacterial Inoculants for Sustainable Agriculture and the Environment

2

Yoav Bashan, Luz E. de-Bashan, and S.R. Prabhu

Abstract

Plants have been inoculated with plant growth-promoting microorganisms to enhance crop yield and performance over four decades. The two central aspects for success of inoculation are the effectiveness of the bacterial strain and the application technology. This chapter discusses characteristics of ideal carriers for bacterial inoculants [plant growth-promoting bacteria (PGPB) and plant growth-promoting rhizobacteria (PGPR)] and focuses on superior formulations for the future, mainly polymeric and encapsulated formulations and new emerging ideas in the field of inoculation. Future research avenues are highlighted.

Keywords

Inoculants • Plant growth-promoting bacteria • PGPR • PGPB • Rhizobia

2.1 Introduction

Inoculation of plants to enhance yield of numerous crops and growth performance is an old practice. Two main factors control the success of inoculation: effectiveness of the bacterial isolate and application technology.

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In general, inoculant technology, especially with plant growth-promoting bacteria (PGPB and its biocontrol section commonly known as PGPR), has marginal impact on productivity in developing countries. This happens because inoculants are not used, are of poor quality, or are homemade (Bashan 1998; Bashan et al. 2014; Calvo et al. 2014). Probably as a result of the potential for cheaper production of inoculants by small companies, compared to expensive chemical fertilizers and pesticides dominated by the giant agro-industries in developed countries, many practical studies of numerous crops were done successfully in developing countries, showing its potential (1st, 2nd, 3rd, and 4th Asian PGPR conferences, http://www.asianpgpr.com, retrieved December 28, 2015). Most of these studies originated in the Indian subcontinent, Vietnam, with cereals and legumes in Latin America, mainly in Argentina and Mexico, and in Africa (Johri et al. 2003; Roy et al. 2015; Cong et al. 2009; Fuentes-Ramirez and Caballero-Mellado 2005; Diaz-Zorita and Fernandez-Canigia 2009; Hartmann and Bashan 2009; Atieno et al. 2012; Mathu et al. 2012). The inoculant market in developed countries had a market value of US$293 million in 2013, with a projected 9.5 % compounded annual growth rate.

The reason for using formulated inoculants is straightforward. Shortly after suspensions of bacteria are inoculated directly into the soil without a proper formulation, the bacteria population of most species of PGPB/PGPR is decimated. This result, combined with poor production of bacterial biomass, difficulty sustaining bacterial activity in the rhizosphere, and the physiological state of the bacteria at application time, can prevent the buildup of a sufficiently large PGPB population in the rhizosphere. A threshold number of viable cells, which differ with species, are essential to obtain the intended positive plant response.

The inherent heterogeneity of any soil is the key obstacle in inoculation. Introduced bacteria sometimes can find all the niches in the rhizosphere colonized by other microorganisms. These unprotected, introduced bacteria must compete with the often better-adapted native microflora and mostly cannot withstand predation by soil microfauna. As a response, a major role of any formulation is to provide a more suitable microenvironment, combined with physical protection for a prolonged time. Formulations employed in the field should be designed to provide a reliable source of bacteria that can survive in the rhizosphere and become available to crops when needed (Herrmann and Lesueur 2013; Bashan et al. 2014; Calvo et al. 2014). Although this is the main purpose of inoculant formulation, many inoculants failed to do this. A recent review pointed out the shortage of using existing biotechnological techniques for inoculant production and formulation (Vassilev et al. 2015).

In the last decade, several reviews summarized the field of plant inoculation. Most concentrated on specific genera, such as *Rhizobium* and *Azospirillum*, field performance of several PGPB, availability of various PGPBs and their modes of action, reduction in the use of fertilizers by including inoculants, and potential marketing (Stephens and Rask 2000; Catroux et al. 2001; Deaker et al. 2004; Herridge 2007; Bashan et al. 2004; Bashan and de-Bashan 2010, 2015; Pereg et al. 2016; Rizvi et al. 2009; Lodewyckx et al. 2002; Andrews et al. 2003; Vessey 2003; Lucy et al. 2004; Adesemoye and Kloepper 2009; Mathre et al. 1999; Berg 2009; Keswani

et al. 2014). Recently, in a whirl of announcements, several large agrochemical industries and microbiology-based companies declared their overlapping interests in developing microbial-based products to improve plant performance without genetic manipulations of the plants. This showed the confidence that major agribusiness and chemical companies have in the potential growth of this industry. Since 2012, multiple acquisitions, licensing agreements, and partnerships with face values worth of hundreds of millions of US dollars show the depth and breadth of investment that large companies are making in microbial product development (Fox 2015; Olson 2015). Even with all the renewed interest and investments in microbial products, the challenges of formulations, quality, and field performance are yet to be tackled (Herrmann and Lesueur 2013; Bashan et al. 2014).

Because of the common unclear literature mix between inoculants, biofertilizers, biopesticides, carriers, and formulations, in this review, "bacterial isolates" refer to specific bacterial strain of PGPB/PGPR that can promote plant growth after inoculation. "Carrier" refers to the abiotic substrate (solid, liquid, or gel) that is employed in the formulation process. "Formulation" refers to the laboratory or industrial process of unifying the carrier with the bacterial strain. "Inoculant" refers to the final product of formulation containing a carrier and bacterial agent or consortium of microorganisms.

2.2 The Ideal Inoculants

When designing an inoculant, the farmers' and the manufacturers' requirements must be considered, which are mostly complementary but not the same. In practice, and above all, farmers always seek maximum yield of their crops. The main practical features of inoculants expected by farmers are that the inoculant has to be compatible with routine field practices, such as seed disinfection and the common use of pesticides. Other important features of inoculants are (1) ease of use, (2) compatibility with the seeding equipment at the time of seeding, (3) tolerance of unintentional abuse during storage, (4) ability to work under different field conditions and types of soil, and (5) ability to help prolong survival of the inoculated bacteria for the time needed by the plant. The additional requirements of manufacturers from the same inoculant are (6) shelf life that lasts more than one growing season, (7) reproducible yield results in the field, and (8) human, animal, and plant safety required by laws. The last item is achieved by eliminating hazardous materials.

The marketplace for inoculants does not have any international standards for quality. In practice, inoculant quality can be under governmental regulations, as in the Netherlands, India, China, Thailand, Russia, Canada, France, Australia (voluntarily), and Argentina (since 2016), or leave product quality to the discretion of the manufacturer, as is common in the USA, Mexico, and the UK, claiming that the market will decide if a product survives. This ambiguity in the 1980s–1990s led to inadequate performance of commercial PGPB inoculants and subsequent abandonment of their use on a global scale (Stephens and Rask 2000; Catroux et al. 2001). Inoculants made a strong comeback since 2000, concentrated in Latin America

(Fuentes-Ramirez and Caballero-Mellado 2005; Diaz-Zorita and Fernandez-Canigia 2009; Hartmann and Bashan 2009) and Southeast Asia, mainly in India (Nguyen et al. 2003; Selvamukilan et al. 2006; Reddy and Saravanan 2013; Roy et al. 2015).

The three fundamental and essential characteristics for inoculants are to (1) support the growth of the PGPB/PGPR, (2) support the necessary number of viable microbial cells in good physiological condition for an acceptable time (Stephens and Rask 2000), and (3) deliver enough microorganisms at the time of inoculation to reach a threshold number that is usually required to obtain a response, i.e., the inoculant must contain enough viable bacteria after the formulation process (Date 2001).

In practice, the formulated carrier (inoculant) is the sole delivery vehicle of live microorganisms from the factory to the plants in the field. The carrier is the major portion (by volume and weight) of the inoculant. Carriers of inoculants can be divided into five categories: (1) peat, coal, biochar, clays, and inorganic soil; (2) waste plant materials of diverse industrial and agriculture origins; (3) inert materials, polymers, and treated rock fragments, such as vermiculite and perlite; (4) plain lyophilized microbial cultures and a mix of oil and dried bacteria (these preparations can later be incorporated into a solid carrier or used as they are); and (5) liquid inoculants, where some chemical is added to the liquid medium containing the PGPB to improve stickiness, stability, surface tension, function, and dispersal (Bashan et al. 2014; Bashan 1998).

Two different formulation types of inoculants exist, i.e., sterile or non-sterile. A sterile carrier has significant advantages of delivering the right microorganism at the precise concentration, avoiding the unpredictable potential for the indigenous organisms to suppress cell counts. Therefore, there is more control over inoculum potential; however, the sterilization process renders the inoculant far less cost-effective, especially in developing countries. Sterile and more pricy inoculants have been successfully marketed in the USA, Australia, Canada, Mexico, and Argentina.

Formulation is the crucial issue for commercial inoculants. This industrial process can determine the commercial success or failure of a biological agent that has outstanding performance under research conditions. Formulation is the industrial "witchcraft" of converting a promising laboratory-proven microorganism, cultivated by skilled specialists in carefully designed and supervised greenhouse experiments into a commercial product used by farmers under diverse and uncontrolled field conditions.

Chemical formulations in agro-products have high standards for long shelf life, ease of use, and resistance to abuse by farmers. PGPB/PGPR inoculants are expected to match them. Yet inoculants must overcome two major problems common to all living microorganisms: (1) loss of viability during storage in the farmer's warehouse and (2) long shelf life and stability at temperatures ranging from −5 to 30 °C.

Regardless of the formulation, the consistency of the inoculants can be liquid, slurry, granular, or powder (Bashan et al. 2014). The raw material of the carrier and the type of formulation vary greatly. The raw material carriers for most commercial inoculants are cheap and naturally abundant. Beside the most common material,

peat, other materials have been proposed, including bagasse, animal manure, alfalfa powder, coir dust (coco peat), biochar, perlite, rock phosphate, charcoal, and a range of coals, lignite, talc, and inorganic soil fractions, mainly clays (Bashan 1998; Stephens and Rask 2000; Bashan et al. 2014).

The five categories of desirable general characteristics for a good formulation are listed here (Bashan et al. 2014):

(1) *Chemical and physical characteristics*: The carrier of a contaminant-free inoculant should be nearly sterile or cheaply sterilized, as chemically and physically uniform as possible, of consistent batch quality, of high water-holding capacity (for wet carriers), and suitable for many bacteria species and strains of PGPB/PGPR. Consistency and availability of raw material are an absolute requirement for all carriers. Because the carrier is a major ingredient of the inoculant production, when varied, established quality control process of such inoculants cannot be adjusted for every batch of raw material during industrial production of an inoculant.
(2) *Manufacturing qualities*: Inoculants must be easily manufactured and mixed by the microbial fermentation industry. It should allow addition of nutrients, have an easily adjustable pH, and be made of a reasonably low-priced raw material with adequate supply and availability.
(3) *Farm-handling qualities*: A major concern is ease of handling, providing rapid and controlled release of bacteria into the soil, and application with standard seeding equipment. This is fundamentally important because farm practices seldom change to accommodate a new technology that delivers a high-quality inoculant with specialized machinery, especially in conservative farm areas (Date 2001).
(4) *Environmentally friendly characteristic*: Complying with contemporary environmental laws over substances that may change soil characteristics, the inoculant should be nontoxic and biodegradable, leave no carbon footprint, and be nonpolluting. Application should minimize environmental risks, such as the dispersal of cells of PGPB/PGPB to the air or groundwater.
(5) *Long-storage quality*: The inoculant must have a sufficient shelf life. One or 2 years at room temperature are often necessary for successful integration into the agricultural distribution system in developed countries (Catroux et al. 2001; Deaker et al. 2004).

Naturally, no single inoculant can have all these capacities at top-end quality. However, a good inoculant should have as many of these characteristics at a reasonably good quality. Synthesizing "super-inoculants" or finding a polymer used in more expensive industries, such as pharmaceuticals, nanotechnology, or cosmetics to accommodate all the desired features is theoretically feasible (John et al. 2011; Schoebitz et al. 2013b). So far, these formulations are produced in laboratories and have not employed in commercial products. So far, no effort to synthesize a carrier with defined superior characteristics for agricultural and environmental purposes has been reported, presumably because of high cost.

2.3 Inoculant Formulations

The performance of an inoculant is often the Achilles heel for commercialization. A microbial strain may function optimally under skillful personnel and precise laboratory conditions; yet, formulating this microorganism into an affordable product used by farmers, where similar results under real field condition are expected, is a difficult task and failure is common (Stephens and Rask 2000; Bashan et al. 2014). The literature describes many tested inoculants (Bashan 1998; Bashan et al. 2014; Calvo et al. 2014), but commercial inoculants appear in only a very few variations.

2.3.1 "Primitive" Inoculants: Raw Culture Media with No Additional Formulation

The old method of inoculating seeds and plants with bacterial culture suspension, as done since the pioneering times of plant inoculation four decades ago, still prevails today. It is a common practice among researchers because it is the least laborious and most described method in the literature.

2.3.2 Liquid Inoculants

Liquid inoculants are an improvement of "no-formulation" inoculants to address some of the limitations listed above. Basically, they are simple microbial cultures or suspensions amended with substances that may improve stickiness, stability, and dispersal abilities (Singleton et al. 2002; Bashan et al. 2014). The main advantage of these inoculants over solid inoculants is that they are easy to handle by farmers. These inoculants are very common and the preferred commercial inoculants specifically for the PGPB *Azospirillum* in the developed countries.

2.3.3 Inoculants Using Organic Carriers

Without doubt, peat is the main carrier for rhizobia in North and South America, Europe, and Australia and the main ingredient of inoculants that is sold in large volumes. It is also suitable for most other PGPB/PGPR. Yet, peat is rarely available and expensive in most of Asia and Africa. All other carriers proposed for PGPB/PGPR–rhizobia are compared with the standard peat carrier. Performance of peat-based inoculants and its shortcomings were intensively and continuously reviewed, as well as details of production of variants. Currently, technical details of the basic peat-based inoculant such as grain size, pH, optimal moisture, other amendments, quality of inoculants, quality control standards, and occupational health and safety are common knowledge (Stephens and Rask 2000; Catroux et al. 2001; Date 2001; Deaker et al. 2004, 2011; Xavier et al. 2004).

Alternatives to peat-based inoculants, popular in the 1980s–1990s, were lignite, charcoal, coir dust, composts of various origins and compositions, sugarcane filter mud, bagasse, soils mixed with various organic amendments, and vermiculite. Most were considered inferior to peat as a carrier (Bashan 1998; Singleton et al. 2002). Some organic inoculants made of some waste materials were tested with success in recent years, mainly in developing counties (Ben Rebah et al. 2007). Some were tested on a large scale and others only had inoculant production reported but not evaluation in situ (Hale et al. 2014). Examples for these formulations were described by Bashan et al. (2014). Although some organic wastes can perform equally well or better than peat as a carrier, the main limitation is the availability of sufficient raw material for an industry. Compost made from cork, bagasse, sawdust, brewery waste, or banana leaves can sustain a small, local inoculant industry where the materials are available. They cannot form the base for a large industry, especially when the raw material batch is variable.

2.3.4 Inorganic and Partly Organic Inoculants

Inorganic inoculants can be made from natural inorganic materials, natural polymers, or synthetic materials. Apart from polymeric inoculants, inorganic inoculants are the oldest version of inoculants (Bashan 1998), and a few such as clay and biochar (300 °C burnt carbon) are experimentally used for reforestation in semiarid zones and as a potential carrier for PGPB/PGPR and carrier for processing polluted water with bacteria (Hale et al. 2014; Schoebitz et al. 2014; Stelting et al. 2014). While most of these inoculants are used on a small scale for crop production, all polymeric inoculants, as far as we know, are experimental. Yet, because they open a new approach to formulation with endless industrial variations, these inoculants are described and discussed in detail in this chapter.

2.3.5 Polymeric Inoculants

Synthetic formulations based on a large variety of polymers have been continuously tested for decades because they offer substantial advantages over peat and better options for industrial production (Table 2.1). These include far longer shelf life, appropriate survival in the field, sufficient cell density, ease of manufacturing, and improved performance of plants in general (Bashan 1998; John et al. 2011; Bashan et al. 2014). So far, for agricultural and environmental uses, these polymers include alginate, agar, kappa carrageenan, pectin, chitosan, bean gum, and several proprietary polymers.

Table 2.1 A sample of polymeric formulations used for producing inoculants of plant growth-promoting bacteria for plants from 1998 to 2015

Formulation	Additives or treatment	Microorganisms used	Plant species or substrate	References
Alginate	None	*Azospirillum brasilense; Azospirillum* combined with *Methylobacterium* sp.	Tomato	Bashan et al. (2002), Yabur et al. (2007), and Joe et al. (2014)
Alginate	None	*Azospirillum brasilense*	Several desert trees	Bashan et al. (2009a, b, 2012)
Alginate	Organic olive residue	*Azospirillum brasilense* and *Pantoea dispersa*	*Pinus halepensis*	Mengual et al. (2014)
Alginate	None	*Azospirillum brasilense; A. lipoferum; Pseudomonas fluorescens; Bacillus megaterium; Serratia marcescens; Enterobacter* sp.	Wheat; maize	Bashan and Gonzalez (1999), Bacilio et al. (2004), El-Komy (2005), Bashan et al. (2006), Schoebitz et al. (2013a, c), Ben Farhat et al. (2014), and El-Gamal et al. (2015)
Alginate	None	*Agaricus bisporus* (champignon)	*Agaricus bisporus*	Friel and McLoughlin (1999)
Alginate	None	*Chlorella vulgaris, C. sorokiniana* together with *Azospirillum brasilense, Bacillus pumilus*, or *Phyllobacterium myrsinacearum; Synechococcus elongatus* together with *A. brasilense*	Tertiary wastewater treatment	Gonzalez and Bashan (2000), Gonzalez-Bashan et al. (2000), Lebsky et al. (2001), de-Bashan et al. (2002, 2004), (2005), (2008), de-Bashan and Bashan (2004, 2008), Hernandez et al. (2009), Perez-Garcia et al. (2010), Covarrubias et al. (2012), Cruz et al. (2013), and Ruiz-Güereca and Sánchez-Saavedra (2016)

(continued)

Table 2.1 (continued)

Formulation	Additives or treatment	Microorganisms used	Plant species or substrate	References
Alginate	None	*Pseudomonas fluorescens*	Sugar beet	Russo et al. (2001)
Alginate	None	*Pseudomonas striata; Bacillus polymyxa (PSB); Azospirillum brasilense*	None	Viveganandan and Jauhri (2000) and Cortés-Patiño and Bonilla (2015)
Alginate	None	*Glomus deserticola (AM mycorrhizae); Yarrowia lipolytica (PS-yeast)*	Tomato; faba bean	Vassilev et al. (2001) and Morsy (2015)
Alginate	None	*Pseudomonas putida*	Corn; velvet leaf	Gurley and Zdor (2005)
Alginate	None	*Rhizobium* spp.	*Leucaena leucocephala*	Forestier et al. (2001)
Alginate	Pea protein	*Bacillus subtilis*	*Brachypodium distachyon* and *Phleum pratense*	Gagné-Bourque et al. (2015)
Alginate	Kaolin, starch, talc	*Streptomyces* sp.	Tomato	Sabaratnam and Traquair (2002)
Alginate	Bentonite	*Raoultella planticola*	None	He et al. (2015)
Alginate	Attapulgite	*Pseudomonas* sp.	None	Wang et al. (2014)
Alginate	Starch	*Raoultella terrigena, Azospirillum brasilense*	None	Schoebitz et al. (2012)
Alginate	Gelatin	*Bacillus subtilis*	None	Tu et al. (2015)
Alginate	Starch	*Streptomyces* sp.	Shrub *Rhamnus lycioides*	Mengual et al. (2016)
Alginate	Humic acid	*Pseudomonas putida, Bacillus subtilis, B. megaterium, Azospirillum lipoferum*	Lettuce; rice	Rekha et al. (2007), Reetha et al. (2014), and Sivakumar et al. (2014)
Alginate	Maltodextrin	Diverse nitrogen-fixing bacteria	None	Campos et al. (2014)
Alginate	Peanut oil	*Beauveria bassiana*	Red fire ants	Bextine and Thorvilson (2002)
Alginate	Skim milk	*Bacillus subtilis* and *Pseudomonas corrugata*	Maize	Trivedi et al. (2005)

(continued)

Table 2.1 (continued)

Formulation	Additives or treatment	Microorganisms used	Plant species or substrate	References
Alginate	Glycerol, chitin	*Pantoea agglomerans*	None	Zohar-Perez et al. (2002)
Alginate	Chitin, bran	None	Cabbage, basil, radish, wheat	Sarrocco et al. (2004)
Alginate	None	*Raoultella planticola*	Cotton	Wu et al. (2014)
Chitosan	None	Several PGPB	Tomato	Murphy et al. (2003)
Carrageenan	None	*Azospirillum brasilense*	None	Cortés-Patiño and Bonilla (2015)
Liquid culture	Alginate	*Sinorhizobium meliloti*	Alfalfa	Rouissi et al. (2014)
CMC/corn starch	MgO	Rhizobia; *Gluconacetobacter diazotrophicus; Herbaspirillum seropedicae; H. rubrisubalbicans; Azospirillum amazonense;* and *Burkholderia tropica*	Cowpea, sugarcane	Júnior et al. (2009) and da Silva et al. (2012)
HPMC	None	*Azotobacter chroococcum*	None	Rivera et al. (2014) and Rojas-Tapias et al. (2015)
Ethyl cellulose; modified starch	Silica	*Pseudomonas fluorescens*	None	Amiet Charpentier et al. (1998)

For earlier studies, see Bashan (1998). *CMC* carboxymethyl cellulose, *HPMC* hydroxypropyl methylcellulose

All these polymers share several basic requirements:

(1) Nontoxic in nature and free of harmful preservatives that affect bacteria within the inoculant and later the inoculated plants
(2) Slowly degradable in the soil by soil microorganisms, thereby gradually releasing the bacteria in needed quantities, usually at the time of seed germination and emergence of seedlings, leaving no secondary pollution
(3) Provide significant physical protection for the bacteria from soil competitors and many environmental stresses (Zohar-Perez et al. 2003; Covarrubias et al. 2012; Cruz et al. 2013)
(4) Hold sufficient moisture for survival of the bacteria
(5) Dispersible in water to allow movement of the bacteria from the polymer to the plants, when necessary

More beneficial features are that these polymeric inoculants (1) can be stored dried at various ambient temperatures for extended time without refrigeration, (2) offer a consistent batch quality for manufacturing and at the same time a better environment for the bacteria, (3) can be industrially manipulated to fit the needs of specific PGPB/PGPR, and (4) can be further amended with nutrients to improve short-term survival of the bacteria upon inoculation, which enhances the success of the inoculation process. This last feature is especially essential for associative PGPB competing in the rhizosphere with native microbes.

The major drawback of polymeric inoculants is that the raw materials for most polymers are relatively expensive, compared to peat, soil, and organic inoculants, and require further expensive handling by the industry at costs similar to those in the fermentation industry. As a direct result, so far no commercial polymeric inoculants are currently available. Yet, these inoculants may represent the future technology. A positive aspect of polymeric inoculants is that they are still the domain of research laboratories and does not have proprietary protection of private companies; hence, relatively more information is available in the scientific literature.

2.3.6 Encapsulated Formulations

The encapsulation of live microorganisms in polymers (also known as immobilization when one microorganism is used and co-immobilization when more than one organism is used) is currently experimental in the fields of agricultural and environmental bacteria inoculation technology. The fundamental industrial concept underlying immobilizing microbial cells is to entrap live microorganisms into a polymeric matrix while maintaining their viability and capacities. The encapsulated final product (bacteria–polymer) is then fermented in a bacterial growth medium for different industrial products concentrating in the production of organic acids, amino acids, enzymes, vitamins, and environmental applications, including bioremediation of toxic materials. The desired bacterial products are taken from the fermenter while fermentation continues. Primarily, immobilized microbial cells are easy to produce, store, and handle during industrial production. The main goal of industrial immobilizations is to maintain the immobilized cells in an active form, at high concentrations, for as long as possible. Any premature release of the microorganisms from these immobilized substrates is undesirable. These industrial formulations are not the topic of this chapter and can be reviewed elsewhere (Gibbs et al. 1999; Kourkoutas et al. 2004).

Immobilized PGPB/PGPR formulations for agricultural and environmental applications have at least two very distinctly different purposes from those of the fermentation industry: (a) They have to provide temporary physical protection for the immobilized PGPB/PGPR in the soil from stressful environmental conditions, microbial competitors, and mini-predators, all hostile to any change in the biological makeup of the soil, and (b) for successful root colonization, they have to release the PGPB/PGPR strain gradually. Liberation of the immobilized bacteria from

the beads occurs when the polymer is slowly degraded by the native soil microorganisms and in the process releasing the PGPB–rhizobia to the soil where plants that need inoculation are growing.

2.4 Macro- and Micro-formulations of Alginate

Through 2015, alginate derivatives are the preferred polymer for immobilization of microorganisms. The alginate formulations are used for various purposes: application of biocontrol agents and mycoherbicides, immobilization of cellular organisms and enzymes, increase of stability of recombinant plasmids in host cells, bacterial chemotaxis research, and primary polymer in formulations of drugs. Alginate is a naturally occurring polymer available worldwide, mainly from different marine macroalgae in large and sustainable quantities (Draget et al. 2002; Yabur et al. 2007), as well as from several bacteria (Sabra et al. 2001; Trujillo-Roldan et al. 2003). The preparation of beads containing PGPB/PGPR is fairly easy and straightforward and involves a multistep procedure at room temperature with minimal amounts of additional chemicals and equipment; thus, it is very popular in research. Procedures to formulate PGPB/PGPR in alginate are available (Bashan et al. 2002; de-Bashan et al. 2004, 2015; de-Bashan and Bashan 2010; Bashan and de-Bashan 2015). Occasionally, biomass of the immobilized PGPB/PGPR strain is low for the specific application; thus, a second multiplication of the immobilized PGPB/PGPR in the already-formed beads is necessary. This step is fairly simple (Bashan 1986). The advantages of alginate formulations are their nontoxic nature, biodegradability, availability at low costs (in 2015, US$ 2 per kg: Chinese product), slow release of the entrapped microorganisms into the soil that can be designed and controlled by variation in the polymeric structure, and approval for human use by the US Federal Food and Drug Administration (Bashan et al. 2002; Zohar-Perez et al. 2002).

2.4.1 Macro-alginate Beads

The technology of macro-alginate beads (1–4 mm diameter) was used to immobilize several PGPB/PGPR and mycorrhizal fungi. For example, *Streptomyces* sp. was formulated in an alginate–kaolin (aluminum silicate) carrier. Initially, the bacteria were mixed with the kaolin, then mixed with alginate, and formed into beads. Finally, the formulation was freeze-dried. This dry form was further formulated as wettable powder by adding starch, talcum, and more kaolin to improve survival of *Streptomyces* sp. in the inoculant for up to 14 weeks. These formulations yielded large variations in biocontrol efficacy of the fungal pathogen *Rhizoctonia solani* in tomato (Sabaratnam and Traquair 2002).

Encapsulation of the PGPB/PGPR *Bacillus subtilis* in alginate beads supplemented with humic acid yielded high viability of immobilized bacteria, with excellent survival after storage for 5 months. Slow release of bacteria from the bead was shown for 1 week at various levels of pH. Successful promotion of lettuce by the

encapsulated bacteria was demonstrated (Young et al. 2006). The success of this particular immobilization technique was attributed to the dual benefits of humic acid for the microbes and plant and the chemical properties of the humic acid, including easy mixing with alginate without interfering in the formation of the alginate gel beads. One benefit of humic acid in the structure of alginate bead is that it serves as a carbon source for encapsulated *Pseudomonas putida* and *B. subtilis*, which promotes survival of the encapsulated microorganisms during storage (Rekha et al. 2007). Immobilization of an encapsulated phosphate-solubilizing bacteria *Serratia* sp. was superior to a non-immobilized inoculant of the same strain on wheat plants (Schoebitz et al. 2013c). The effectiveness of free and encapsulated PGPR *Raoultella planticola* in promoting cotton growth under saline stress demonstrates that encapsulated inoculants have more positive effects on cotton seedlings than free cells (Wu et al. 2014). The efficacy of wet macrobeads of an alginate/starch mix was demonstrated when several *Streptomyces* spp. were inoculated onto a perennial shrub under revegetation conditions in the field in semiarid environment (Mengual et al. 2016).

An extended survival time in dry alginate bead was demonstrated when two PGPB, *Azospirillum brasilense* and *Pseudomonas fluorescens*, immobilized in two types of alginate bead inoculants recovered after being dried and stored at ambient temperature for 14 years. Although the populations in the beads had decreased, significant numbers survived (10^5–10^6 CFU g^{-1} beads). After inoculating wheat plants in a growth chamber, both species colonized and enhanced growth equal to those that had not been stored (Bashan and Gonzalez 1999). Vassilev et al. (2001) demonstrated that tomato plants inoculated with an AM fungus (*Glomus desert-icola*) and phosphate-solubilizing yeast (*Yarrowia lipolytica*) that were co-immobilized in alginate are a useful technique for establishing plants in nutrient-deficient soils. Several plant growth parameters were equal in treatments whether the tomatoes were inoculated with the free AM fungus or alginate-entrapped fungus, but inoculation with the fungus and the yeast produced better results.

White mushroom (*Agaricus bisporus*) cultivation was improved with wet alginate inoculant applied to spawn, providing a shorter adaptation (lag) period and higher growth rate in pasteurized compost, compared to liquid spawn and conventional commercial grain spawn. Superiority of this delivery system is attributed to high biomass loading capacity of the beads, protection of the mycelia in the bead microenvironment, and even spatial distribution of beads in the compost (Friel and McLoughlin 1999).

Protection against high temperatures can be provided by alginates. Alginate formulation supported high populations and survival of the phosphate-solubilizing bacteria *Pseudomonas striata* and *Bacillus polymyxa* at storage temperatures of 40 °C (Viveganandan and Jauhri 2000). Several macrobead alginate formulations of *B. subtilis* and *Pseudomonas corrugata* were found superior over liquid inoculants or charcoal-based inoculants for improving maize growth under low temperatures in the Indian Himalayas (Trivedi et al. 2005). Survival of the rhizobacteria *Raoultella terrigena* and *A. brasilense* during encapsulation can be improved by incorporating

starch in bead composition and using trehalose, a disaccharide, in growth culture medium (Schoebitz et al. 2012).

Another application of macro-alginate bead inoculants is in tertiary wastewater treatment by microalgae (de-Bashan and Bashan 2010; de-Bashan et al. 2015). A combination of microalgae (*Chlorella vulgaris* or *C. sorokiniana*) and microalgae growth-promoting bacterium *A. brasilense* was co-immobilized. This unique system removes phosphorus and nitrogen nutrients from municipal wastewater. Co-immobilization of the microalgae and the bacterium provided superior results in experiments over several years to remove these nutrients than the microalgae used alone (de-Bashan et al. 2002, 2004; Hernandez et al. 2006; Covarrubias et al. 2012; Cruz et al. 2013). Co-immobilization of the cyanobacteria *Synechococcus elongatus* with the bacterium *A. brasilense* removed more phosphorus from aquaculture wastewater than cyanobacteria cells that were immobilized alone (Ruiz-Güereca and Sánchez-Saavedra 2016). Leftover debris from wastewater treatment that contain alginate beads with the two microorganisms were dried and stored for a year and then used to improve growth of sorghum and enhance eroded desert soil fertility (Trejo et al. 2012; Lopez et al. 2013). Similarly, the combination of co-immobilized *A. brasilense* and *C. vulgaris* improved the growth of tomato plants under saline condition (Escalante et al. 2015).

While alginate formulation may have solved difficulties associated with common peat inoculants (Bashan 1998), the application of macro-alginate beads as inoculants has two major disadvantages. (1) An additional treatment during sowing is needed even if the inoculant is planted by the seeding machine. In developed countries, the grower who is already too busy during sowing may be short of time and reluctant to incur additional expense and time. In developing countries, the farmer might not inoculate the seeds at all. The root of this problem is insufficient agricultural education and conservative cultural traditions that make some small-scale farmers suspicious of new technologies, especially those involving live bacteria. (2) The bacteria released from the inoculant needs to migrate through the soil toward the plants. Under typical agricultural practices, when beads are loosely mixed with seeds and sown together by planters, the beads might fall up to a few centimeters from the seeds. The bacteria released from the beads must move through the soil, facing competition and predation by the native microflora, often more aggressive and better adapted to the soil than the added PGPB/PGPR. Sometimes the absence of a continuous film of water, essential for such movement, is a limiting factor. These distances, large on a microbial scale, might prove prohibitive for many added PGPB/PGPR, even *Azospirillum*, with proven motility in soil (Bashan and Levanony 1987; Bashan and Holguin 1994).

2.4.2 Alginate Microbeads

The microbead (50–200 μm in diameter or smaller) was developed to overcome the two fundamental difficulties of macrobeads. The idea considers that, if the beads are small enough but capable of encapsulating a sufficient number of bacteria, it would

be possible to produce a "powder-like" formulation similar to powdered peat inoculants. The seeds are coated with bead powder at the seed-handling facility and sold to the farmer as "improved seeds." Coated seeds with fertilizers, fungicides, or hormones are commonplace and accepted by most farmers. In developed countries with large-scale agricultural practices, pre-coated seeds eliminate an additional field treatment and provide the most convenience for growers and incentive to use. This significant benefit notwithstanding pre-coating seeds with PGPB/PGPR is not an easy industrial task, considering all the other amendments mentioned above. Some are toxic or not compatible with PGPB/PGPR. Consequently, so far it has been done only on a small experimental scale. Yet, a similar formulating idea, but with a peat inoculant, has been applied commercially for a long time as a pre-inoculation of forage legumes, such as alfalfa. The peat containing the PGPB/PGPR is applied to the seeds as slurry. Later an adhesive is added, and finally inoculated seeds are covered with finely ground calcium carbonate (Brockwell 1977).

The production of alginate microbeads is relatively simple and involves low-pressure spraying through a small nozzle, resulting in small-diameter droplets of an alginate solution mixed with liquid bacterial culture suspended in a very rich medium. These droplets, while sprayed into a slowly stirred solution of $CaCl_2$, immediately solidify into microbeads at diameters ranging between 100 and 200 μm, which entrap ~10^8–10^{10} CFU g^{-1} bacteria, similar to the population levels entrapped in alginate macrobeads (Bashan et al. 2002; Bashan 1986; Campos et al. 2014). Specialized equipment is available (Bashan et al. 2002), and commercial microbead equipment is already available. An alternative and more complicated technique to produce microbeads is that the size of calcium alginate gel beads can be controlled by applying high voltage that affects the size of the alginate solution droplets from a few millimeters to a few 100 micrometers, as voltage is increased. The droplets are then conventionally hardened with $CaCl_2$. So far, this last idea has not been applied to any useful microorganisms, apart from baker's yeast (Murakata et al. 2001). A simple alternative might be milling of dry solid sheets of alginate containing PGPB/PGPR into a powder and using an agricultural adhesive for coating seeds (Fig. 2.1).

Application of microbead alginate formulations to inoculate plants in soils includes: (1) using several transplanted desert tree species and cacti in desert reforestation programs. These successful long-term shade house (8 months) and field experiments (11 years, to 2015) used the PGPB/PGPR *Azospirillum brasilense* and phosphate-solubilizing *B. pumilus* entrapped in microbeads, where the inoculant was added to the planting holes beneath the root balls (Bashan et al. 2009a, b, 2012). (2) A field assay in a semiarid environment to assess the influence of inoculation with a mixture of two immobilized strains of PGPB/PGPR (*A. brasilense* and *Pantoea dispersa*) and olive residue on the growth of Aleppo pine *Pinus halepensis* showed that 28 months after planting, the microbial inoculation was the most effective treatment for stimulating seedling growth and absorbing nutrients. The inoculated plants had low accumulation of proline, less oxidative damage of lipids, and higher potential of water in the shoots (Mengual et al. 2014). (3) *P. fluorescens* was immobilized in 300–700 μm alginate microbeads; the survival of the

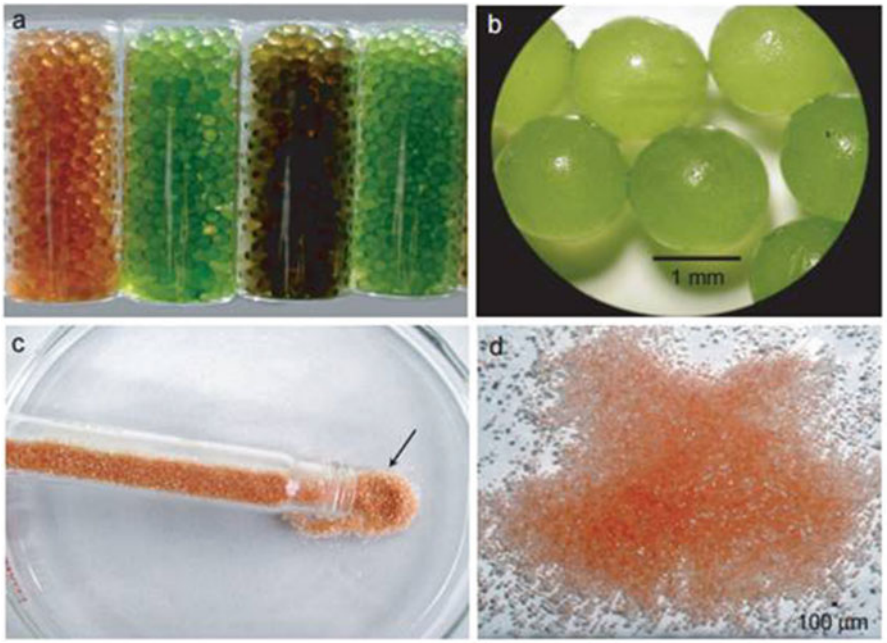

Fig. 2.1 Immobilization of microorganisms in alginate inoculants. (**a**, **b**) Wet macrobead inoculant and (**c**, **d**) dry microbead inoculant. (**a**) Inoculants of (from *left*) *Azospirillum brasilense*, *Chlorella vulgaris*, *C. vulgaris* (in alginate obtained from *Sargassum*), *C. sorokiniana*. (**b**) Macrobeads containing an association between *A. brasilense and C. sorokiniana*. (**c**, **d**) Dry alginate microbeads of *A. brasilense* (*arrow*)

microorganisms and ability to colonize sugar beet were measured after 1 year. Although dried alginate beads reduced the quantity of viable bacteria, the microbeads provided a satisfactory level of root colonization and protection against two fungal pathogens *Pythium ultimum* and *Rhizoctonia solani*. The capability of the immobilized bacteria to produce the antifungal metabolite 2,4-diacetylphloroglucinol was not affected after storage for 12 months (Russo et al. 2001). (4) Alginate beads coated with peanut oil, containing the entomopathogenic fungus *Beauveria bassiana*, were used against the red fire ant *Solenopsis invicta*. Broadcast applications and individual mound treatments with this inoculant reduced activity of the ant populations (Bextine and Thorvilson 2002).

2.4.3 Future Improvements of Micro-alginate Beads

Currently, food, pharmacology, nanotechnology, and cosmetics are far larger research fields employing immobilization than agriculture. Consequently, several technical improvements derived from these fields, aiming to make the polymer more suitable for immobilization of biological materials, were proposed. Although

these techniques were unrelated to agricultural/environmental inoculants, they offer insights for future developments (John et al. 2011; Schoebitz et al. 2013b). A few examples having potential for inoculant production are: (1) Biotin was covalently coupled with alginate in an aqueous-phase reaction that combines the advantages of alginate gelling to entrap cells to provide a gentle hydrated and highly porous environment and the high-affinity interaction of the avidin-biotin complex. The conjugate was successfully used to immobilize bioluminescent reporter cells into microbeads (Polyak et al. 2004). (2) Alginate hydrogels were reinforced at the surface with several secondary polymers to enhance mechanical strength and stability to delay degradation in soil (Bashan 1986; Nussinovitch 2010). Common alginate hydrogels were reinforced with polyethyleneimine, leading to greater elasticity than gels without polyethyleneimine. The stable interactions of the alginate and polyethyleneimine prevented alterations of the pore structure in the gels and slowed deterioration of gel properties, even under continuous agitation in a bioreactor (Kong and Mooney 2003). The high stability of barium alginate beads was improved by a multilayer coating with polyethyleneimine and polyacrylic acid (Gaumann et al. 2000). Short-chain alginate was synthesized and used for coating the membranes of microcapsules to provide high mechanical strength (Chang et al. 2002). To extend degradation time and attain maximum mechanical strength, a chitosan-alginate-$CaCl_2$ system was accomplished to produce water-insoluble membranes of biodegradable polymers (Wang et al. 2001). These ideas may need improvements to adapt them to agriculture/environmental practices. Particularly in agricultural applications, water-soluble membranes on the microcapsules are necessary to release microbes because the solvent in the soil is water. (3) A method to form macroporous beads with an interconnected pore structure in alginate was developed to improve growth and survival of microorganisms by incorporating gas pockets within the beads. This stabilized gas bubbles with surfactants and subsequently removed the gas (Eiselt et al. 2000).

In summary, based on experiments in the last three decades, it appears that alginate is the most promising polymer. However, with the relatively limited published research on alginate beads tied to agriculture/environment projects and even if the material is currently inexpensive compared to other polymers, it is premature to predict whether alginate will displace peat in the agro-inoculation industry or will remain in the industrial and environmental microbiology setting, where it runs supreme.

2.5 Polymeric Inoculants with Other Materials

Ironically, although commercial alginate preparations are not yet available for PGPB/PGPR, several other polymers that are used in industrial and environmental microbiology may be considered as substitutes when the microorganisms fail to adapt to alginate preparations. Even though all materials are still experimental, it is worth mentioning them to encourage further research on these potential carriers. Earlier cases are listed in Bashan (1998) and Bashan et al. (2014).

Formulation using chitosan as a carrier for several PGPR was mixed with soilless growth medium for successful biocontrol against cucumber mosaic virus in tomato (Murphy et al. 2003). Five PGPB were prepared into several formulations of polymers composed of carboxymethyl cellulose–starch. These formulations maintained the bacterial strains in high numbers during 60–120 days of storage. The formulations were effective in promoting the growth of sugarcane cuttings (da Silva et al. 2012). Rhizobia that were formulated with the same inoculants kept their populations inside the inoculants during 165 days of storage and were still capable of promoting growth of cowpeas (Júnior et al. 2009). *P. fluorescens* was formulated with three polymers: commercial film-forming "methacrylic acid copolymer" (Evonik Industries, Darmstadt, Germany), ethyl cellulose, and a modified starch. The best performer was the commercial polymer, where bacteria survived for 1 year. Survival of bacteria was related to the microspheres' residual moisture; the highest survival of bacteria occurred when the residual moisture was ~25 %. This inoculant was not tested on plants (Amiet Charpentier et al. 1998). With limited information on these carriers, it is impossible to predict their future as bacterial inoculants.

2.6 Dry Polymeric Carriers

A main mission of immobilizing PGPB/PGPR for agricultural and environmental uses, similar to older inoculant types, is to increase shelf life, rather than maintain a high bacterial count, since the number of microorganisms decreases during storage. From commercial and agricultural perspectives, longer survival of bacteria in these polymeric preparations makes dry formulations extremely attractive.

Several studies tested dry alginate inoculants. A microbead formulation containing *A. brasilense* was air-dried at 38 °C to form a powder; each particle contained $>10^9$ CFU g^{-1} bacteria. Alternatively, dry microbeads were produced using a standard freeze-drying procedure. The dry preparation was easily attached to dry seed surfaces by adding a lecithin or a synthetic adhesive. The bacteria in both inoculants were slowly released from the microbeads in concentrations ranging from 10^4 to 10^6 CFU g microbeads^{-1} d^{-1}, depending on the formulation and the time of incubation. Longer incubation periods led to lower numbers of bacteria being released. The dry inoculant enhanced development (dry weight, height of plants) of wheat and tomato seedlings growing in infertile soil and was biodegraded within 15 days in moist soil (Bashan et al. 2002). A similar microbead formulation used for desert reforestation with leguminous trees was air-dried in flat trays at 30 °C for 24 h without losing efficacy. The resulting effect lasted for several years in the field (Bashan et al. 2009a, b, 2012). The efficacy of freeze-dried alginate beads was tested with an agricultural strain of *Pantoea agglomerans*. The dry beads were produced with bacteria supplemented with glycerol and chitin. Glycerol increases pore size within the beads, which affects the slow-release properties, where addition of glycerol and chitin enhanced survival during the freeze-drying process. These beads were able to protect the applied PGPB/PGPR to the soil compared to bacterial suspension

(Zohar-Perez et al. 2002). Dry seed coats of various formulations of alginate, either alone or with bran and chitin additives, but not containing a PGPB, did not affect the viability or percent of germination of seeds of wheat, basil, cabbage, and radish (Sarrocco et al. 2004), but Na alginate supplied as an additive to liquid formulation without polymerization significantly extended the shelf life of *Sinorhizobium meliloti* (Rouissi et al. 2014). Dry beads (by hot temperature) made of alginate–bentonite were very efficient in slow release of the PGPR *Raoultella planticola*, but this formulation was not tested on plants (He et al. 2015). Other combinations of alginate with pea protein of *B. subtilis* protect the cells in the soil; the PGPR was capable of colonizing two model plants (Gagné-Bourque et al. 2015).

2.7 Shelf Life of Inoculants

Liquid inoculants produced in the field by in situ fermenters and immediately applied are uncommon, and only a few exist for turf grass for golf courses and hydroponic cultivation. For conventional agricultural applications, inoculants made of peat or other organic and inorganic materials are used, and a designated shelf life between manufacturing and application is usually required. The shelf life of inoculants for more than one growing season, while retaining its biological traits intact, is a major challenge for any formulation. So far, the most common solutions to this problem of extending survival time have been to (1) reduce moisture in the formulation and produce dry formulation by drying in either a fluidized bed, air-drying, or freeze-drying. These processes reduce water content in the final product or (2) store under refrigeration. In completely dry formulations, bacteria remain in a dormant form, its metabolism is very slow or even halted, and in this form, they are resistant to environmental stresses, are insensitive to contamination, and are more compatible with fertilizer applications.

The main difficulty with dry polymeric formulations is the survival of the microorganisms from the three stresses they have to endure: immobilization stress, desiccation, and rehydration before application on plant roots. During stress and storage, the mortality rate reaches >90 % of the initial incorporated population from the fermenter. The dehydration phase is perhaps the most critical and most stressful for microbes during the formulation process. This is particularly difficult for nonspore-forming Gram-negative bacteria, which are the majority of species among PGPB/PGPR. Additional stress occurs when reviving the bacteria at the time of inoculation, which produces hydration stress on the cells. Survival in formulated inoculants is affected by several factors: the culture medium used for cultivating the PGPB/PGPR, physiological state of the bacteria when harvested from the medium, process of cell immobilization, use of protective materials, type of drying procedure, and speed of dehydration. Drying during formulation is a crucial step. The highest death rate occurs either soon after manufacturing, while in storage, or immediately after application to the seeds or soil (Date 2001). Yet, if properly dehydrated, shelf life of the dried formulation is much longer than any liquid, wet, or moist inoculant.

A comparison between two PGPB/PGPR showed that using various organic, inorganic, and polymeric formulations, *Bacillus subtilis* survived at room temperature (~22 °C) for 45 days, but *P. putida* required refrigeration (~0 °C) and depends on the type of carrier that is used (Amer and Utkhede 2000). When γ-ray irradiated cork compost or perlite inoculants, with zero contamination, were stored at 25 °C, rhizobia in these inoculants remained unchanged for 90–120 days of incubation; inoculants composed of two clays maintained a high bacterial population for more than 5 months (Albareda et al. 2008). This is also typical for polymeric inoculants that are essentially sterile.

The commercial literature we found on the internet agrees that shelf life of 1–2 years under warehouse conditions for peat inoculants is desirable, even though, in practice, this is not true for many contemporary PGPB/PGPR inoculants. Storage time is even longer for polymerized and synthetic inoculants. Longer period of storage was tested on PGPB/PGPR. Dry alginate beads containing *A. brasilense* stored for 1 year at room temperature retained significant growth promotion effects on sorghum plants even though the populations of *A. brasilense* within the dry beads declined with time (Trejo et al. 2012). Identical data was found in a dry alginate–starch inoculant, where 76 % of viable cells of *A. brasilense* survived more than 1 year of storage (Schoebitz et al. 2012). Survival time of 3 years, without losing viability of the entrapped bacteria, occurred in wet alginate beads at 4 °C for *B. subtilis* and *P. corrugata* (Trivedi and Pandey 2008). The longest survival time, without losing efficacy, was 14 years for *A. brasilense* and *P. fluorescens* in dry alginate beads (Bashan and Gonzalez 1999).

In summary, a practical formulation must maintain enough viable bacteria over acceptable periods of time to ensure successful seed inoculation. Longer shelf life can be obtained by either increasing the number of microbes in the inoculant, so despite a decline in population over time, enough cells remain alive at seeding time. Alternatively, use an additive in the formulation to increase growth during storage or maintain cold storage that reduces the rate of decline in bacteria. In this case, even formulations with lower starting populations can be acceptable (Xavier et al. 2004). Yet, polymerized dry formulations are far superior for extending shelf life.

2.8 Emerging Innovative Products: Cell-free Microbial Products

The inherent limitations of live cell-based inoculants are still a strong bottleneck in widespread application of bioproducts and continue to delay commercialization and use of PGPB/PGPR on a larger scale. Evidently, there is growing interest to produce and market bioproducts that are not affected by soil and weather conditions and are capable of consistent performance. “Inoculants” made of active fragments and metabolites of PGPB/PGPR in the absence of living cells are emerging. Many elicitors of plant defenses and secondary metabolites of PGPB/PGPR are documented. These microbial products, if produced commercially, have potential as “inoculants”

for reducing agrochemical use (Compant et al. 2013). There are growing numbers of commercial, cell-free microbial products based on metabolites, which can be integrated with growers' standard practices and are generally not affected by soil or weather conditions. These products are based on the assumption that all beneficial microbes act on plants through bioactive molecules (Mabood et al. 2008; Zhuang et al. 2013). Microorganisms have been the source of some highly active biomolecules of significant commercial importance, and, due to their chemical complexity, biological fermentation processes remain the best means of production (Warrior 2000).

Several natural products from microbes, such as harpin proteins, have found commercial applications in improving crop health (Copping and Duke 2007). Lipochitooligosaccharides are nodulation factors secreted by rhizobia that induce nodules on the roots of legumes (Truchet et al. 1991). Although lipochitooligosaccharides are important signal molecules for plant–symbiont interactions, they can directly impact general nonlegume plant growth and development (Prithiviraj et al. 2003; Tanaka et al. 2015). Potential commercial lipochitooligosaccharide products for seed and foliar applications in legumes and nonlegumes, such as corn, are currently available in North America. Concentrated metabolites containing lipochitooligosaccharides, exopolysaccharides, and hormones produced from rhizobia have a shelf life of 24 months. This product improved yields of soybean and corn and is currently registered for use in Brazil (Marks et al. 2013). Lipochitooligosaccharides are known to improve plant symbiosis with vesicular–arbuscular mycorrhizal fungi (Xie et al. 1995). More practical uses of this chemical are expected in coming years.

2.9 Future Prospects

Even though the prevailing opinion in many new companies using PGPB/PGPR is that formulation is a side issue and quick to develop once the right PGPB/PGPR is selected, realistically, developing new and effective formulations for inoculation of PGPB/PGPR and rhizobia is a very slow process and consumes resources. Creation of new formulations is a challenge in practical microbiology, and shortcuts usually lead to failure of the inoculant in the field. Improvements in formulations are key to the development of enhanced high-end inoculants. Literature surveys show that the identification of new isolates having PGPB/PGPR capacities is often not difficult, and many are identified annually. Yet, development of most PGPB/PGPR strains stops there, without ever reaching the formulation stage.

While several thousand articles in the literature describe inoculation of plants with PGPB/PGPR, only a handful focused on delivery systems. Mostly, formulations and application techniques, if described at all, are hidden within the Materials and Methods section of publications on other topics. Grant applications in the developed countries regarding formulations are rarely successful. This topic, which spans fundamental microbiology and industrial technologies done simultaneously in

research facilities and commercial agricultural fields, is mostly neglected. For the future of plant inoculation, this topic cannot be ignored.

The following research topics should be top priorities of research on new or improved delivery systems for PGPB/PGPR and rhizobia, excluding peat formulations, which have already reached their peak:

- In-depth evaluation of known carriers. Periodically, a new formulation, involving a known carrier, such as alginate or liquid inoculant containing a supplement, is presented as a solution to all the maladies of previous carriers. In-depth analysis of the pros and cons for each formulation is seldom investigated and, if done by the industry, is mostly unpublished. Because numerous carrier materials of inoculation practices were proposed in recent decades, fair and honest evaluation of the most common formulations, liquid, organic wastes, and polymeric, is needed.
- Improvement of formulations that showed positive field results needs fine-tuning of key ingredients (quantities, conditions, ratio of mixtures) or improvements in the process of production. Although evaluation is assumed to be done by the industry, specific information is not available.
- Improve survival of the PGPB/PGPR in the inoculant. Reducing the decline of the population of the PGPB/PGPR during formulation and application should be a major target of research. If cell mortality can be significantly reduced, it may be possible to raise the number of cells applied per seed by two or three orders of magnitude. This includes physiological age of cells (growth phase) and relative humidity and water activity during storage, which is species dependent. Slow drying is usually superior to fast drying. Lower water activity brings better survival because it is a restraint on survival, especially at high temperatures. Optimizing the rate of rehydration should be a research target.
- Shelf life is an essential commercial concern because application time in the field is short, while production time in the factory of large quantities is long and usually cannot be done close to application time. Current shelf life is relatively short or too short. Maintaining efficacy for 2 years is optimal. Yet, various experimental formulations, such those involving alginate, show that PGPB/PGPR survival, without losing efficacy for several years, is achievable. This objective deserves better attention, even though it takes years to obtain the data.
- Multi-strain inoculants and combination of inoculants containing rhizobia and PGPB/PGPR. Numerous studies have shown the advantages of these combinations, usually demonstrated only in the laboratory without any formulation. While formulation of several microorganisms does not add additional significant technical difficulties compared to formulation of one microorganism, the interaction of the partners within these formulations is largely unknown. Development of such consortia inoculants require explorations regarding (1) compatibility between microbial populations, (2) symbiosis or interaction with plants, (3) efficiency of their resulting plant growth-promoting effects, (4) their growth rate when together, (5) potential biofilm formation, and (6) technical difficulties of culturing microorganisms in a fermenter, where each has different nutritional

requirements or other in vitro cultivation conditions (Reddy and Saravanan 2013). Two (or more) different microorganisms grown (and formulated) separately or mixed inoculant that are developed after a mixed fermentation can be developed. The former would be called "compounded inocula" while the latter "complex inocula." As multi-strain applications appear to be the current frontier for PGPB/PGPR (Vassilev et al. 2015), appropriate formulation should be explored.

- Additives to many formulations are given. However, studies of additives used in formulations have been done ad hoc and are largely empirical. Seldom is their mode of action understood. This part of the formulation process is a virgin field and deserves more attention.
- Polymeric inoculants. Even though many studies pointed out that this is the future of inoculants, no such formulation for PGPB/PGPR or rhizobia has passed the threshold of industrial approval. Immobilization of microorganisms is a large emerging field in pharmaceutical, nanotechnology, medicine, aquaculture, and cosmetics. Many different and efficient immobilization techniques were developed for those purposes [Schoebitz et al. 2013b; Bioencapsulation Innovations (http://bioencapsulation.net)]. Almost none of these technologies were tested in the inoculant field apart from simple polymerization of a few polymers. Many of these emerging technologies from other fields merit testing in the agricultural inoculant industry.
- It is doubtful that commercial farming practices will significantly change, even to accommodate a technology that delivers a high-quality inoculant. Consequently, the goal should be to create formulations that are farmer friendly, as some of the contemporary inoculants are. The best approach will be those formulations which do not require additional effort by the farmer.
- Agriculture and common environmental uses cannot use inoculants with high production costs or expensive carrier materials. Consequently, because large quantities of inoculants are used for staple food crops, cereals, legumes, and not more expensive cash crops, any inoculation technology must be developed with low costs in mind. It is highly unlikely that an outstanding formulation with a high price will find a niche.
- Local strains should be used for improved performance because no PGPB/PGPR strain can perform best under all farming conditions. Since the effectiveness of inoculation depends on multiple factors, including the target plant species and soil and weather conditions, inoculants, in theory, should also be differentiated and matched appropriately for ever-changing cultivation conditions. This situation complicates the task of providing effective inoculants because production economics dictates keeping the variety of inoculants small. This industrial requirement is contrary to the reality of diversity among crop species, inoculant species, climates, and soil biotic communities that otherwise support the production and distribution of multiple inoculants.
- For transplanted crops, inoculation in the nursery is far simpler and usually yields better results. The cost of product and its application is far less because the

volume of the inoculant is small and growth conditions are easier to control. Hence, more research and transfer of technologies should be targeted to nursery-grown plants. For example, seedling roots dipped in microbial inoculants without formulation are effective and easy for microbial inoculation of transplanted rice (Choudhury and Kennedy 2004). This treatment needs to be modified and applied to other transplanted crops.

- A number of PGPR produce lipopeptides with biosurfactant activity (Raaijmakers et al. 2010) that directly inhibit pathogens and provide systemic-induced resistance in crops (Ongena et al. 2007; Ongena and Jacques 2008). There are prospects of developing cell-free lipopeptide product biofungicides where part of the efficiency of the product is due to lipopeptides supplied along with live cells. More exploration of microbial metabolites is required to obtain more benefits from such PGPB/PGPR.
- Microbiome studies produced so far only fundamental knowledge regarding the complexity of the microbial world. Beneficial microbial strains capable of beneficially changing soil microbial community structure have been isolated (de-Bashan et al. 2010a, b; Kang et al. 2013; Lopez et al. 2013). However, this fundamental information is yet to serve as a basis for the next generation of inoculants (Berg et al. 2013, 2014; Massart et al. 2015).
- Information regarding formulation and application techniques should be precisely described in the literature. Specifically, each new manuscript should contain the precise formulation of the inoculants in quantitative details, including all non-active materials and active supplements. In cases where the inoculant is proprietary or is intellectual property, the serial registration number of the patent or the intellectual property and the country of registration should be disclosed. All bacterial species and variants, not just the genus, should be disclosed and be available from microbial collections that are available to the public. When strains are intellectual property of an organization, the name of the organization holding the rights should be disclosed. If specific sequence(s) of a strain is publically known, this should be disclosed as the definitive identification of the strain. All microbial strains in a consortium must be listed. Inoculation techniques should be described in detail sufficient to allow repetition of the experiment (Bashan et al. 2016).

In summary, formulation and field application of inoculants are a pure technological platform that is based on fundamental principles of microbiology and material sciences. The joining of these fields creates useful products that will continue to be an important input in sustainable agriculture and remedial environmental solutions.

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Dedication This chapter is dedicated to the memory of the Israeli soil microbiologist Prof. Yigal Henis (1926–2010) of the faculty of Agriculture, the Hebrew University of Jerusalem in Rehovot, Israel, one of the pioneers of studies of inoculants in Israel.

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Formulation and Commercialization of Rhizobia: Asian Scenario

3

Rajendran Vijayabharathi, Arumugam Sathya, and Subramaniam Gopalakrishnan

Abstract

The symbiotic agreement of rhizobia with leguminous plants is making a valuable contribution to agriculture primarily as nitrogen fixers and secondarily as plant growth promoters by their key role as phosphate solubilizers, growth hormone producers, abiotic and biotic stress relievers, and host-plant resistance enhancer. In the so far identified 14 genera and 105 species of rhizobia, a huge number of research reports were reported in various aspects. Genetically modified rhizobia with desirable traits have also been surfed to a large extent. Besides their potentiality, the commercial success of rhizobia as a bio-inoculant is poor, because most of the inoculants produced worldwide are of poor or suboptimal quality. Though voluminous data and better understanding are available on various formulation technologies, longevity and efficacy of the final product are loosed at the farmer's end. This book chapter is focused to address various types of formulations applicable to rhizobia, quality control for longevity, gaps in knowledge on bringing the native potential of rhizobia during formulation, and critical control points to be considered during its development. The chapter also shares ICRISAT's experience in its rhizobial collection, formulation developments, and efficacy testing.

Keywords

Rhizobia • Legumes • Asia • Formulations • Peat • Legislations

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3.1 Introduction

Approximately 80 % of the human dietary nitrogen needs, i.e., 24 Tg/year in tropics and subtropics, are satisfied by the plants. But with the increasing earth's population at a rate of 1.4 % annually, the present scenario of crop production rates will not be sufficient to maintain the dietary needs (Mannion 1998; Fink et al. 1999). Deterioration of agricultural lands and use of marginal lands for crop production are further complicating the scenario, because soil N management plays a critical role in crop yield (Huang and Rozelle 1995; Bramley et al. 1996; Rozelle et al. 1997; Savant et al. 1997). While considering the past scenario, i.e., between 1950 and 1990, N fertilizers played a major role in increasing the cereal grain yield. They yielded 6–9 mg grain/hand take-up 200–300 kg N ha year^{-1} (Vance 1998). Still the use of N fertilizers at global scale is in increasing trend as per the FAOSTAT data. Though nitrogen fertilizers gave an increase in crop production, there was a great impact in the environment which includes NOx loss, acid rain, higher leaching, change in the global N cycle, and polluted ground water. When developed countries were facing such problems, developing countries were affected by the additional issues of fertilizer cost, availability, and distribution problems (Kinzig and Socolow 1995; Vitousek et al. 1997).

In the context of sustainable N management, symbiotic nitrogen fixation (SNF) plays a vital role. Though it represents systems including either rhizobia, *Azolla*, or *Anabaena* with either leguminous or cereal crops, the system of legume-rhizobia symbiosis is the critical factor as it involved in 80 % (approximately 100–122 Tg year^{-1}) of biologically fixed nitrogen by involving a range of species such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Allorhizobium* (Vance 1998; Herridge et al. 2008). The process of nitrogen fixation through SNF was reviewed periodically at various aspects covering biochemical and molecular mechanisms and genetic factors (Jiao et al. 2016; Remigi et al. 2016). When considering the fixed nitrogen effect by fertilizer and SNF, two key factors have to be considered: (i) fixed N by SNF is less susceptible to volatilization, leaching, or denitrification than fertilizer N, and (ii) industrial production of N requires approximately 1.5 Kg oil Kg^{-1} fertilizer in order to reduce N to ammonia along with the requirement of high temperature and pressure. Though SNF is also an energy-demanding process involving 16–24 moles of ATP for reducing 1 mole of dinitrogen, its persistence, stability, and absence of post-fixation effects add positive impact over fertilizer N. As per the review by Herridge et al. (2008), symbiosis by rhizobia is the efficient system for SNF as it contributes 55,140 kg N ha^{-1}, whereas 0.330 kg N ha^{-1} is by other biological systems. The symbiosis by cyanobacteria contributes for 5 Tg N, whereas by free-living, associative, and endophytic bacteria provides 10–20 Tg N. Actinorhizal symbiosis estimates about 4–42 g N tree^{-1} (Dommergues 1995), and cycads contribute 8–19 kg N h^{-1} in a year (Vessey et al. 2004).

Rhizobia, the efficient nitrogen fixer, are a term used for collective bacteria that enters symbiosis with legumes. Initially, till 1982, it was considered that *Rhizobium* is the only bacteria that possess these properties, but today, it was identified that there are 14 genera in two subphyla of Proteobacteria, viz., α-Proteobacteria and

β-Proteobacteria. α-Proteobacteria includes the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, *Shinella*, and *Sinorhizobium* (syn. *Ensifer*), and β-Proteobacteria includes *Burkholderia*, *Cupriavidus*, and *Herbaspirillum*. The number of genera in the rhizobia list is increasing day by day by various studies. This increasing number of rhizobia isolation led to reclassification and redesignation of some species (Lindström et al. 2010).

Development of such rhizobia as inoculants for legume crops is the most valuable contributions ever made by science to agriculture since it is evident to reduce N fertilizer use. Initial studies of inoculation were performed at a very basic level and laborious moving of soil from fields of well-nodulated legumes to legume-free fields (Fred et al. 1932). European countries initiated the inoculums development by advising their farmers to treat legume seeds with glue and sieved air-dried soil from well-nodulated plants (Walley et al. 2004). The work of Hellriegel and Beijerinck in the 1880s has brought a record on using pure cultures of rhizobia on inoculation of legume seeds. Within a couple of years, rhizobia were available in the European market for a range of species, and still it is getting developed involving new technologies (Guthrie 1896; Perret et al. 2000). But in the context of Asian countries, still the legume inoculant technology is underdeveloped due to a range of factors. Hence, this book chapter is focused to discuss the factors affecting rhizobia inoculant development in Asia.

3.2 Beneficial Traits of Rhizobia

Rhizobia are primarily considered for nitrogen fixation. Still the research on SNF in relation to rhizobia is ongoing including genetically modified rhizobia (Lindström and Mousavi 2010; Okazaki et al. 2016). After the concept of plant growth-promoting rhizobacteria by Kloepper, rhizobia have also been surfed to a large extent for its plant growth-promoting (PGP) properties (Kloepper and Schroth 1978). Hence, a developed rhizobial inoculum will provide additional plant and soil health benefits besides fixing nitrogen. PGP properties of rhizobia have been reviewed previously by Gopalakrishnan et al. (2014) and Naveed et al. (2015). The representatives of rhizobia with PGP traits have been given here.

3.2.1 Rhizobia as Phosphate Solubilizers

Rhizobia including *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Mesorhizobium mediterraneum*, *Bradyrhizobium* sp., and *Bradyrhizobium japonicum* (Vessey 2003; Afzal and Bano 2008) are the potential P solubilizers. The solubilization was aided by low molecular organic acids produced by them, for instance, 2-ketogluconic acid production by *R. leguminosarum* (Halder et al. 1990) and *R. meliloti* (Halder and Chakrabarty 1993). Enhanced growth in chickpea and barley plants by P-solubilizing rhizobia *M. mediterraneum* has been demonstrated by Peix et al. (2001).

3.2.2 Rhizobia as Iron Mobilizers

Iron exists as insoluble hydroxides and oxyhydroxides which cannot be accessed by both plant and microbes. Some bacteria synthesize low molecular weight compounds termed as siderophores which are capable of sequestering Fe^{3+}. Many rhizobia species including *R. meliloti*, *Rhizobium tropici*, *R. leguminosarum*, *Sinorhizobium meliloti*, and *Bradyrhizobium* sp. are reported to be potent siderophore producers (Arora et al. 2001; Carson et al. 2000).

3.2.3 Phytohormone Production of Rhizobia

Phytohormones are the essential substances for plant growth stimulation. They include indole-3-acetic acid (IAA), cytokinin, and gibberellins. IAA is the foremost phytohormone and plays a role in cell division and differentiation and also in nodule formation. Rhizobia strains are also reported to produce IAA via indole-3-pyruvic acid and indole-3-acetaldehyde pathway (Camerini et al. 2008). Similarly rhizobia have been reported to produce cytokinins which are involved in root development and root hair formation (Senthilkumar et al. 2009). Gibberellins which are responsible for stem elongation and leaf expansion are also reported in *Rhizobium* (Boiero et al. 2007). Some reports are there for production of abscisic acid which stimulates stomatal closure, induces seeds to store proteins, and induces gene transcription for protease inhibitors (Dobbelaere et al. 2003).

3.2.4 Rhizobia as Biocontrol Agents

Biocontrol properties have been demonstrated in several rhizobia strains through the mechanisms like competition for nutrients (Arora et al. 2001), production inhibitory substances including antibiotics (Chandra et al. 2007), production of hydrolytic enzymes (Ozkoc and Deliveli 2001), siderophores (Carson et al. 2000; Deshwal et al. 2003), and low molecular weight metabolites (Bhattacharyya and Jha 2012). Phytopathogens such as *Rhizoctonia solani*, *Macrophomina phaseolina*, and *Fusarium solani* were found to be controlled by rhizobia.

3.2.5 Rhizobia as Abiotic Stress Relievers

The stress of the plant depends on host-plant reaction which can be influenced by rhizobia and the symbiosis (Yang et al. 2009). Several reviews periodically documented the stress tolerance of *Rhizobium* and *Bradyrhizobium* against soil salinity, acidity, alkalinity, osmotic stress, and temperature fluctuations (Graham 1992; Kulkarni and Nautiyal 2000; Grover et al 2010).

3.3 Development of Rhizobia Formulations

Development of an inoculant technology for microbes is a time-consuming and cumbersome process as it faces various issues because many of the microbes produce fruitful laboratory results but fail to reflect similar effects under field conditions. So the success of an inoculant depends on its optimal results in situ and sophisticated use including cost-benefit ratio by end user (Xavier et al. 2004). In the context of inoculant development, carrier, a vehicle which transfers the microbes from laboratory to field, plays a crucial role. An ideal carrier should provide a beneficial microenvironment for the inoculated microbes against a range of biotic and abiotic stress factors including contaminants, soil antagonists, soil health deterioration, temperature, dryness, UV light, and mechanical stress. It should include the features such as (1) sustained availability, (2) low cost, (3) high moisture absorption capacity, (4) easy to process, (5) easy to sterilize, and (6) buffering capacity (Keyser et al. 1993). An overview on the available carrier materials and different types of inoculants is given in Fig. 3.1. Each carrier and formulation technology has its own pros and cons; and several reviews summarizing the same are available (Jung et al. 1982; Van Elsas and Heijnen 1990; Daza et al. 2000; Catroux et al. 2001; Amarger et al. 2001; Deaker et al. 2004; Bashan et al. 2014; Nehra and Choudhary 2015; Gopalakrishnan et al. 2016). Different rhizobial formulations tested on various crops are summarized in Table 3.1.

America, Europe, and Australia have potential market for rhizobia and have well-developed inoculant technologies. It is estimated that, in Australia, legumes

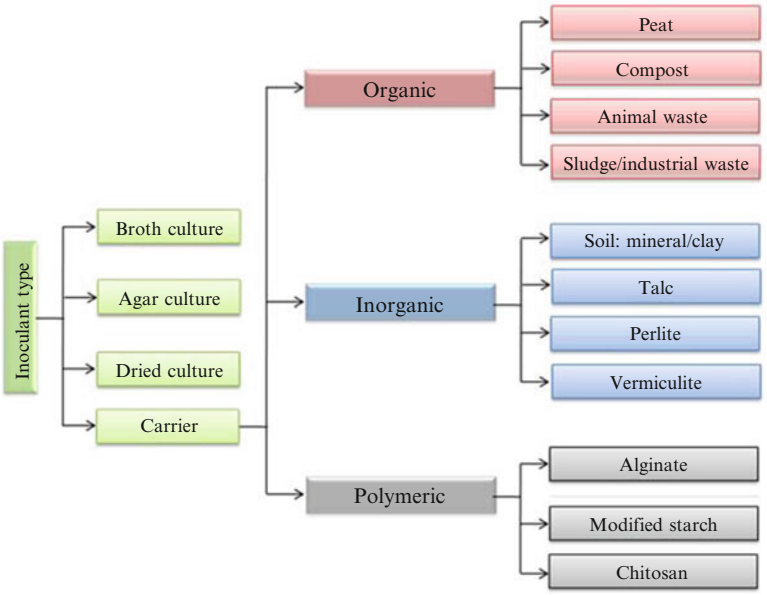

Fig. 3.1 Overview of inoculant types

Table 3.1 Different formulations of rhizobia tested at field levels

Formulation types	Additive/ treatment	Rhizobia	Crop tested	References
Liquid (culture media or water)	Glycerol, PVP, trehalose, FeEDTA	*B. japonicum*	Soybean	Singleton et al. (2002)
	PVP; FeEDTA	Several rhizobia; *B. japonicum*	Soybean	Albareda et al. (2008)
	Unknown (commercial)	*B. japonicum*	Soybean	Maurice et al. (2001)
	Gum Arabic	*Bradyrhizobium* sp., *Rhizobium* sp.	*Acacia mangium*, green gram, *Leucaena leucocephala*	Diouf et al. (2003); Wani et al. (2007)
Lyophilized cells	Soybean oil/ peanut oil	*Rhizobium* sp.	Bean, cowpea, peanut	Kremer and Peterson (1983)
Organic carrier				
Peat	None or with undisclosed additives	*B. japonicum*; *Rhizobium* sp., *R. leguminosarum* bv. *viciae*	Chickpea; faba beans; maize; pea; soybean; wheat	Clayton et al. (2004a, b), Hamaoui et al. (2001), Hungria et al. (2010), Hynes et al. (2001), Khalid et al. (2004), and Revellin et al. (2000)
	Gum Arabic	*Rhizobium*, *Bradyrhizobium*	Bean, *Lupinus*, *Hedysarum* Soybean	Albareda et al. (2009) and Temprano et al. (2002)
Coir dust/coco peat	None	*Azorhizobium caulinodans*	Rice	Van Nieuwenhove et al. (2000)
Vermicompost/ earthworm compost	Lignite	*R. leguminosarum*	Not tested	Raja Sekar and Karmegam (2010)
Sawdust	Composted by inoculation with *Cephalosporium* sp. and *Azospirillum brasilense*	*B. japonicum*, *R. meliloti*	Groundnuts, lucerne, and grass mixture of bird's foot trefoil and ryegrass; soybean	Kostov and Lynch (1998)
Sawdust	None	*R. leguminosarum*	*Trifolium repens*	Arora et al. (2008)

(continued)

Table 3.1 (continued)

Formulation types	Additive/ treatment	Rhizobia	Crop tested	References
Grape bagasse, cork compost	Gum Arabic, CMC	Several rhizobia; *B. japonicum*	Soybean	Albareda et al. (2008)
Wastewater sludge	Acid, alkaline, and oxidative pretreatments	*S. meliloti*, *R. leguminosarum* bv. *viciae*, *B. japonicum*, *B. elkanii*	Not tested	Ben Rebah et al. (2002a, b)
Inorganic carrier				
Clay minerals, perlite	Gum Arabic, CMC	Several rhizobia, *B. japonicum*	Soybean	Albareda et al. (2008)
Coal	None	*R. leguminosarum* bv. *phaseoli*	Pinto bean	Crawford and Berryhill (1983)
Vermiculite	None	*B. japonicum*, *S. meliloti*, *R. leguminosarum* bv. *phaseoli*	Navy beans	Graham-Weiss et al. (1987) and Sparrow and Ham (1983)
Perlite	Gum Arabic	*Rhizobium*, *Bradyrhizobium*	Bean; *Lupinus*, *Hedysarum*; soybean	Temprano et al. (2002)
	Sucrose	*R. leguminosarum* bv. *phaseoli*, *R. tropici*, *B. japonicum*	Bean, soybean	Daza et al. (2000)
Polymeric carrier				
Alginate	None	*Rhizobium* spp.	*Leucaena leucocephala*	Forestier et al. (2001)

Modified from Bashan et al. (2014)

growing on 25 M ha of land fix US$3–4 billion worth of N annually (Bullard et al. 2005). Report of Vessey (2004) states the benefits of rhizobial inoculants in the Northern Great Plains of the USA and Canada on soybean, lentil, pea, and faba bean with an overall response of 45 % yield increase. In the context of Asia, the situation is typically different though it contributes for maximum production of pulses than the other regions/continents. The statistical data of FAO (FAOSTAT 2016) on top seed producers and fertilizer users clearly indicates that the major seed producers are India and China; also the two Asian countries are the relatively top consumers of fertilizers (Fig. 3.2). All these together give a clear indication that the Asian countries depend more toward N fertilizers than the biofertilizers contributing nitrogen

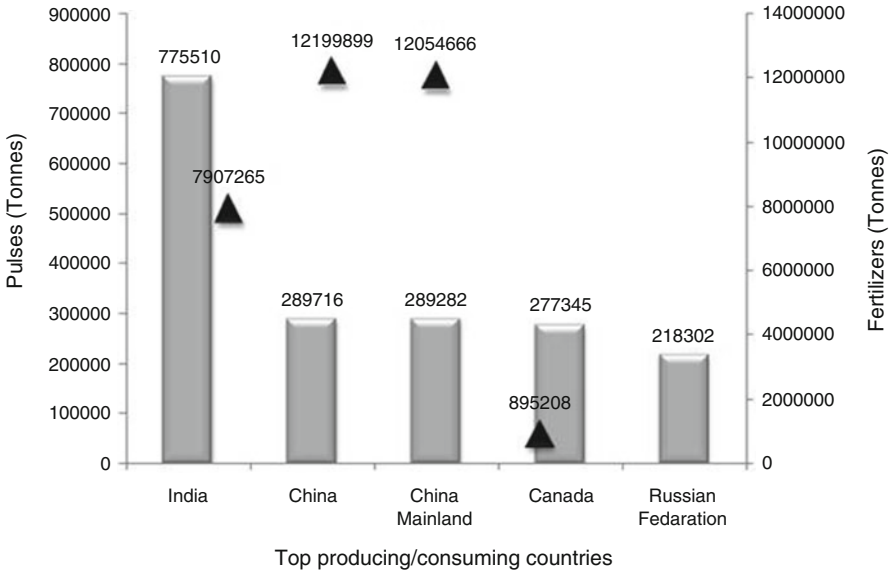

Fig. 3.2 Top pulse producers and fertilizer consumers of the world (Note: ■ Top seed producer (based on average data of 1993–2013) on the left axis ▲ Top fertilizer consumers (based on average data of 2006–2009) on the right axis)

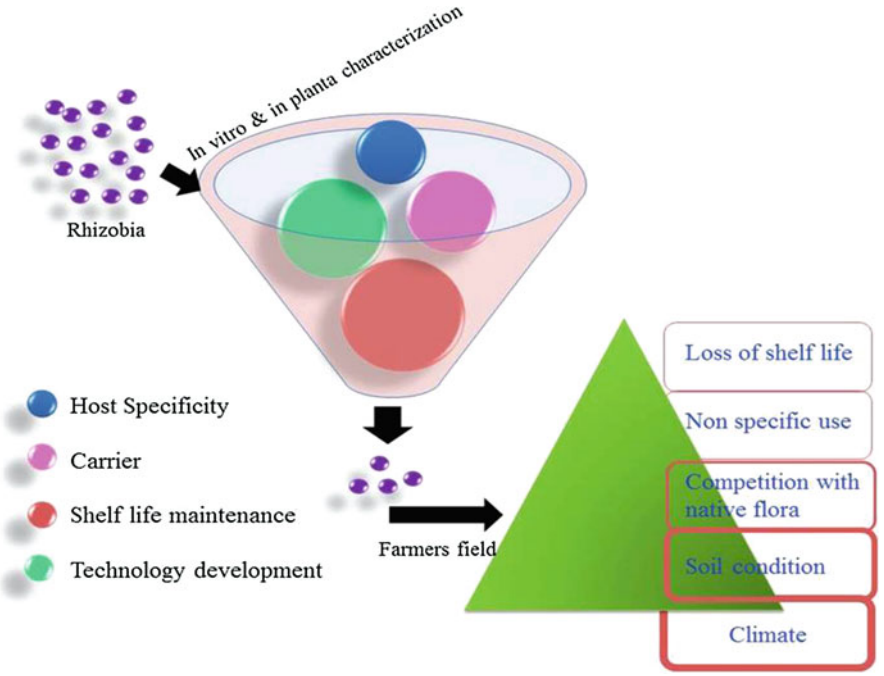

Fig. 3.3 Overview of barriers in inoculant development

including *Rhizobium* inoculants. The problem in the context of Asian scenario in *Rhizobium* inoculant technology is described here in various aspects, and an overview is given in Fig. 3.3.

3.3.1 Inoculant Strain Selection

Effective rhizobial strain is the central core for developing an inoculant which is necessitated in order to provide rhizobia for new legume cultivars and species and extend and optimize the legume cultivation under fluctuating environmental conditions. Brockwell et al. (1995) have listed a set of essential and desirable characters for inoculant strains including host specificity, competence with native rhizobia population and also with agrochemicals, genetic stability, etc. Asian countries including India (Ansari et al. 2014), China (Jiao et al. 2015), Nepal (Adhikari et al. 2012), and Myanmar (Htwe et al. 2015) have been reported with vast diversity of nodulating rhizobia. Recent reports on diversity analysis of rhizobia under hostile environments such as soils with acidity (Mishra et al. 2014), alkalinity (Singh et al. 2016), and micronutrient deficiency (Unno et al. 2015) indicate the research initiatives on the exploration of Asian rhizobial strains. The large genetic diversity noticed on soybean native rhizobia of Asian countries further supports the phenomenon (Biate et al. 2014). Reeve et al. (2015) captured the phylogenetic and biogeographic diversity of root nodule bacteria across the world through two genome sequencing reports, which has only 7 entries for rhizobia from Asian origin among the 107 selected strains. However, these 7 entries include 3 among the total of 13 type strains and 1 among the total of 14 elite strains with commercial significance, indicating that the complete characterization and exploration of rhizobial biodiversity of Asian countries will pave way for inoculant development.

3.3.2 Genetically Modified Rhizobia

Besides the native flora, genetic modification has also been done in rhizobia, mainly to compete with the indigenous strains and to improve its efficacy to form nodules and to fix nitrogen.

3.3.2.1 Modification in Nodulation

To increase the nodulation efficiency, two approaches were carried out. One is by introducing genes encoding for trifoliotoxin, an antibiotic to which indigenous flora is sensitive. Robleto et al. (1998) used this construct in *Rhizobium elti*, the common bean microsymbiont. They differ with the indigenous strain only in the production of nodules. Over 2 years, the genetically modified strains had occupied 20 % of the nodules in comparison to non-trifoliotoxin-producing strains. Another approach is to modify the expression of metabolite *putA* gene which is responsible for root surface colonization. Dillewijn et al. (2001) followed this approach in alfalfa field with *S. meliloti* strains overexpressing *putA* gene. On 1 month of inoculation, a large

number of strains occupied the nodules than the control strains. It appears to be an efficient method of nodulation, but on the yield of crop after 3 years of experimentation, they were all equal in inoculated and un-inoculated plants. This informs that inoculant strains will improve in nodulation only when indigenous competing population is less efficient which might not be frequent.

3.3.2.2 Modification in Nitrogen Fixation

To improve the nitrogen fixation, two approaches were followed. One is involving modification in *nifA* gene which regulates the expression of genes necessary for enzymes involved in nitrogen fixation. The other is by modulating dicarboxylate transport (*dct*) genes which supplies the carbon and energy required for nitrogen fixation. A construct with extra copy of *nifA* and/or *dct* genes was inoculated in *S. meliloti* and released in four fields (Bosworth et al. 1994). There was an increase by 13 and 18 % of alfalfa biomass in wild-type strains and non-inoculated control, respectively. But they were shown only at the sites with very low population of indigenous flora and low nitrogen content. Further, these were not found after 3 years of exploitation (Scupham et al. 1996). A study on soybean cultivation with release of *B. japonicum* with or without extra copy of *nifA* gene did not neither increase the yield nor the nitrogen fixation (Ronson et al. 1990). Summarily, the success of genetic modification has the potential to bring out a success in poor agricultural conditions.

3.3.2.3 Interaction Between Indigenous and Genetically Modified Rhizobia

In response to the introduction of genetically modified rhizobia, there was a change in number, composition, and activities of indigenous microflora and most importantly exchange genetic material with indigenous microflora. There were very less differences observed in rhizospheres of different hosts (Hirsch and Spokes 1994; Amarger et al. 2001) which informs only less changes happen on introduction. Similarly, vice versa transfer, i.e., plasmids from native flora to the introduced flora, was also not detected on re-isolating the genetically marked rhizobia after 1–2 years of introduction (Hirsch 1997). Data predicts that plasmid acquisition takes place at a frequency of 8×10^6/recipient cell in one site after 7 years of release which is not a stable conjugant. Studies have reported that there is no transfer of Tn-7 plasmid that occurs at any stage. If occurred also, the frequency is less than 10^7 events/gram of soil (Drahos et al. 1986). Lilley and Bailey (1997) had reported that transfer from indigenous to genetically marked rhizobia takes place with a frequency from 5×10^7 to 1 per recipient which varies with the year of experiment. However, the generated transconjugant is not stably maintained in the cell.

3.3.3 Nutritional Attributes for Rhizobia

After the selection of effective rhizobia, nutritional attributes have to be considered in order to evaluate whether the given carrier material will be enough to hold the viability or it requires any additional supplements for rhizobial maintenance.

Knowledge of nutritional requirement is a key factor when selecting complex material like agricultural, industrial, and sewage sludge wastes for inoculant production. Broadly rhizobia are divided into two categories depending on nutritional requirement and growth rate. They are fast-growing and slow-growing rhizobia which are placed in the genus *Rhizobium* and *Bradyrhizobium*, respectively (Jordan 1984). Fast growers are acid producers with 2–4 h as generation time. Slow growers are alkaline producers with 6–8 h as generation time (Jordan 1984). Fast growers can grow on various carbon sources such as hexoses, pentoses, disaccharides, trisaccharides, and organic acids (Allen and Allen 1950), whereas the other type can grow only in the presence of pentose but can utilize many aromatic substrates (Parke and Ornston 1984). In the context of nitrogen, some fast growers are potent in utilizing nitrate, ammonia, and amino acids (Quispel 1974). Amino acid glycine, alanine, and certain D-forms of amino acid might create a negative impact in nitrogen fixation (Burton 1979). Vitamin requirements vary between the genera, for example, *R. leguminosarum* (bv. *trifolii* and bv. *phaseoli*) requires biotin, thiamine, or calcium pantothenate separately or in combination, whereas *S. meliloti*, *B. japonicum*, etc. need only biotin (Graham 1963). In case of minerals, deficiency of Ca^{2+} and Mg^{2+} affects the growth and results in abnormal cells (Vincent 1962).

3.3.4 Inoculant Development

Among the inoculants are the primitive types such as broth culture, agar culture, and dried/lyophilized cells. These types of inoculants could not be promoted to practical technology, though it is least laborious and has proved records at research centers, because of impractical application at large scales and its failure to meet economic and commercial needs (Bashan et al. 2014). Hence, a carrier is necessary for the development of a successful inoculant.

The major markets, such as Europe and Australia, supply the inoculants in solid carriers, most commonly peat, for seed application (Catroux et al. 2001; Singleton et al. 2002). However, in North and South America, the inoculants supplied are clay- and peat-based granular and liquid inoculants (Singleton et al. 2002; Xavier et al. 2004). The Asian market also depends on peat for its inoculants because of its potential in holding high numbers of rhizobia (greater than 10^8 cells/g) during the storage. Unlikely, they do not have enough peatlands due to the lack of harmonized policies related to the management of peatlands besides their presence in Indonesia, India, Malaysia, Myanmar, the Philippines, Singapore, Thailand, and Vietnam. In the last few years, a forward look for its sustainable management has arisen. The projects ASEAN Peat l and Forests Project (APFP) and SEApeat were aimed in reducing deforestation and degradation of peatland forests and to strengthen the policies for its management. On the other end, a large area of peatlands in Vietnam has been designated as protected area and national parks (http://www.aseanpeat.net/).

It should also be considered that whether the peat belongs to these regions is original peat. Thomas et al. (1974) have evaluated the physicochemical characters of peat obtained from Nilgiri reserves of India and concluded that the material was

not an original peat as it lacks the main traits like water-holding capacity and organic carbon content. It is noticed that the Indian peat has 20–50 % organic carbon, whereas Australian and American peat has 65 and 86 % organic carbon content, respectively (NIIR 2004). Conservation policies for peatland management by Europe, Australia, and America have become stringent as they have key roles in biodiversity, carbon sequestration, and fuel-related application. This indirectly leads to the unavailability and high export cost for other countries (Joosten 2015).

As an alternative to peat, other organic carriers such as lignite and charcoal can be used which have also proved to be efficient in carrying rhizobia with the shelf life of 4–6 months (Argal et al. 2015; Gao et al. 2015). Research on alternate carrier was started more than four decades ago on carriers such as lignite and coal, clays and mineral soils, compost, farmyard manure, pressmud, agricultural waste, and inorganic materials like vermiculite, perlite, ground rock phosphate, calcium sulfate, polyacrylamide gels, and alginate (Kandasamy and Prasad 1971; Dube et al. 1980; Chao and Alexander 1984; Iswaran et al. 1972; Philip and Jauhri 1984; Sadasivam et al. 1986; Sparrow and Ham 1983; Dommergues et al. 1979; Jung et al. 1982).

There are numerous reports on research and development of successful rhizobial formulations which were tested in fields of various research stations. However, there are very few number of coordinated network projects on large-scale evaluation in Asian countries. On the contrary, the International Network of Legume Inoculation Trials (INLIT) funded by the US Agency for International Development (USAID) in the University of Hawaii's NifTAL project assessed the need for inoculation in tropical agricultural systems by conducting 228 trials on various legumes such as green gram, soybean, black gram, groundnut, cowpea, chickpea, lentil, pigeon pea, and common bean. Worldwide Rhizobial Ecology Network (WREN), the follow-up program of NifTAL, evaluated the factors contributing to variations in inoculation response including a number of infective rhizobia, edaphic characteristics, crop fixed-N demand, and soil fixed-N supply (Singleton et al. 1992).

Effective regulatory quality control (QC) program has key role in the successful production of rhizobial inoculants. This may be supported by appropriate legislation as in Canada, Uruguay, and France or may be voluntary as in Australia, Thailand, New Zealand, and South Africa. Contrarily, in the USA, regulatory control and independent testing are considered unnecessary, with manufacturers conducting their own internal QC. Irrespective of the QC nature, all QC programs should monitor the numbers and quality of the strains in the inoculants along with the contaminating microorganisms. In Asia, 90 % of inoculants sampled had $<10^8$ rhizobia g^{-1} carrier and most of samples were contaminated (Thompson 1992).

Besides these barriers, many Asian countries commercialized rhizobia inoculants. This includes the following: (1) Pakistan, Fasloon Ka Jarasimi Teeka (AARI), BioPower (NIBGE), Biozote (NARC), and Rhizogold (ISES, UAF), consists of *Rhizobium* sp. (Naveed et al. 2015), and (2) Japan, the Tokachi Federation of Agricultural Cooperative (TFAC), produces Mamezo (rhizobia are mixed with peat and the natural organic matters), R-processing seeds (leguminous seeds inoculated with rhizobia), and hyper-coating seeds (leguminous grass seed coated by rhizobia within the capsule of calcium carbonate) (Yokoyama and Ohyama 2007).

Table 3.2 Viability and longevity of five different formulations of six different rhizobia

	Colony-forming unit (CFU/ml) of different carrier materials in months												
	0	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th
	Chickpea													
IC-59														
T1	4.5×10^{9}	3.0×10^{9}	2.4×10^{9}	2.2×10^{9}	2.0×10^{8}	1.5×10^{8}	1.3×10^{8}	1.1×10^{8}	9.1×10^{7}	5.7×10^{7}	2.3×10^{7}	1.2×10^{7}	8.6×10^{6}	3.0×10^{6}
T2	2.0×10^{9}	1.8×10^{9}	1.4×10^{9}	1.2×10^{9}	8.9×10^{8}	7.5×10^{8}	5.4×10^{8}	4.3×10^{8}	1.2×10^{8}	9.0×10^{6}	4.2×10^{6}	1.2×10^{6}	7.5×10^{5}	4.5×10^{6}
T3	2.9×10^{9}	2.0×10^{9}	2.1×10^{9}	1.7×10^{8}	1.2×10^{8}	8.9×10^{7}	6.3×10^{7}	4.8×10^{7}	2.3×10^{7}	1.1×10^{7}	9.7×10^{6}	5.6×10^{6}	2.3×10^{6}	1.1×10^{6}
T4	7.5×10^{8}	4.7×10^{8}	5.5×10^{8}	4.2×10^{8}	1.0×10^{8}	9.3×10^{7}	7.1×10^{7}	5.6×10^{7}	2.8×10^{7}	9.0×10^{6}	7.0×10^{6}	1.1×10^{6}	7.3×10^{5}	2.6×10^{5}
T5	1.9×10^{9}	1.1×10^{9}	1.4×10^{9}	1.1×10^{9}	8.4×10^{8}	8.5×10^{7}	6.7×10^{7}	4.5×10^{7}	2.7×10^{7}	1.7×10^{7}	9.3×10^{6}	4.8×10^{6}	2.5×10^{6}	1.2×10^{6}
IC-76														
T1	3.9×10^{9}	4.3×10^{9}	2.15×10^{9}	1.75×10^{9}	1.3×10^{9}	1.1×10^{9}	1×10^{9}	9.6×10^{8}	5.1×10^{8}	4.3×10^{8}	1.2×10^{8}	1.0×10^{8}	9.3×10^{7}	2.0×10^{7}
T2	3.3×10^{9}	1.8×10^{9}	9.5×10^{8}	5.5×10^{8}	1.1×10^{8}	9.9×10^{7}	7.1×10^{7}	5.0×10^{7}	3.4×10^{7}	2.1×10^{7}	1.1×10^{7}	7.6×10^{6}	2.5×10^{6}	1.0×10^{6}
T3	5.0×10^{9}	2.0×10^{9}	1.1×10^{9}	1.1×10^{9}	6.6×10^{8}	1.1×10^{8}	8.9×10^{7}	6.1×10^{7}	4.9×10^{7}	3.1×10^{7}	1.2×10^{7}	5.6×10^{6}	3.1×10^{6}	1.2×10^{6}
T4	7.0×10^{8}	4.0×10^{8}	2.5×10^{8}	1.5×10^{8}	1.1×10^{8}	7.6×10^{7}	4.3×10^{7}	2.1×10^{7}	8.1×10^{6}	6.1×10^{6}	3.2×10^{6}	1.1×10^{6}	5.5×10^{5}	2.1×10^{5}
T5	2.3×10^{9}	1.1×10^{9}	5.5×10^{8}	4.0×10^{8}	1.0×10^{8}	6.6×10^{7}	3.3×10^{7}	1.1×10^{7}	9.3×10^{6}	3.7×10^{6}	2.3×10^{6}	1.1×10^{6}	7.5×10^{5}	3.5×10^{5}
	Pigeon pea													
IC-3195														
T1	7.6×10^{9}	5.9×10^{9}	3.2×10^{9}	1.7×10^{9}	1.0×10^{9}	9.0×10^{8}	8.3×10^{8}	8.1×10^{8}	7.6×10^{8}	6.3×10^{8}	2.1×10^{8}	1.2×10^{8}	4.1×10^{7}	1.4×10^{7}
T2	4.7×10^{9}	2.6×10^{9}	1.4×10^{9}	7.0×10^{8}	5.9×10^{8}	2.5×10^{8}	1.3×10^{8}	8.7×10^{7}	7.0×10^{7}	5.6×10^{7}	1.1×10^{7}	8.3×10^{6}	3.7×10^{6}	1.3×10^{6}
T3	6.8×10^{9}	1.6×10^{9}	9.0×10^{8}	5.0×10^{8}	4.0×10^{8}	1.7×10^{8}	9.7×10^{7}	5.3×10^{7}	2.1×10^{7}	1.8×10^{7}	9.3×10^{6}	7.6×10^{6}	5.0×10^{6}	1.5×10^{6}
T4	9.5×10^{9}	4.0×10^{9}	2.1×10^{9}	1.1×10^{9}	7.0×10^{8}	2.3×10^{8}	8.9×10^{7}	7.1×10^{7}	6.5×10^{7}	4.0×10^{7}	1.3×10^{7}	8.9×10^{6}	4.2×10^{6}	1.7×10^{6}
T5	8.7×10^{9}	3.4×10^{9}	1.9×10^{9}	1.0×10^{9}	8.0×10^{8}	5.6×10^{8}	2.3×10^{8}	1.0×10^{8}	9.6×10^{7}	7.0×10^{7}	3.0×10^{7}	9.1×10^{6}	5.0×10^{6}	2.1×10^{6}
IC-4062														
T1	9.9×10^{9}	8.5×10^{9}	6.3×10^{9}	2.85×10^{9}	2.3×10^{9}	1.6×10^{9}	8.6×10^{8}	4.7×10^{8}	2.5×10^{8}	1.8×10^{9}	1.1×10^{9}	6.6×10^{7}	4.6×10^{7}	2.1×10^{7}
T2	3.8×10^{9}	2.1×10^{9}	1.6×10^{9}	9.0×10^{8}	4.2×10^{8}	3.2×10^{8}	1.3×10^{8}	1.0×10^{8}	9.1×10^{7}	7.0×10^{7}	3.0×10^{7}	8.6×10^{6}	4.3×10^{6}	1.2×10^{6}
T3	2.1×10^{9}	7.9×10^{9}	6.3×10^{9}	3.6×10^{9}	1.6×10^{9}	1.1×10^{9}	8.8×10^{8}	4.7×10^{8}	3.1×10^{8}	2.4×10^{8}	1.1×10^{8}	7.8×10^{7}	5.2×10^{7}	1.6×10^{7}

(continued)

Table 3.2 (continued)

	Colony-forming unit (CFU/ml) of different carrier materials in months													
	0	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th
T4	2.0×10^{9}	1.7×10^{9}	1.3×10^{9}	8.0×10^{8}	4.0×10^{8}	9.8×10^{7}	7.9×10^{7}	5.5×10^{7}	3.2×10^{7}	1.0×10^{7}	6.9×10^{6}	3.3×10^{6}	1.1×10^{6}	6.8×10^{5}
T5	2.0×10^{9}	2.3×10^{9}	1.8×10^{9}	1.0×10^{9}	6.6×10^{8}	9.1×10^{7}	6.3×10^{7}	4.3×10^{7}	2.7×10^{7}	1.9×10^{7}	9.4×10^{6}	7.8×10^{6}	3.3×10^{6}	1.1×10^{6}
	Groundnut													
IC-7001														
T1	9.4×10^{9}	8.0×10^{9}	7.0×10^{9}	6.3×10^{9}	1.9×10^{9}	1.0×10^{9}	9.2×10^{8}	8.3×10^{8}	6.0×10^{8}	5.6×10^{8}	2.4×10^{8}	1.1×10^{8}	7.9×10^{7}	3.4×10^{7}
T2	8.4×10^{9}	5.8×10^{9}	4.9×10^{9}	3.6×10^{9}	1.5×10^{9}	8.8×10^{8}	5.4×10^{8}	4.8×10^{8}	3.1×10^{8}	2.2×10^{8}	1.1×10^{7}	7.7×10^{7}	3.5×10^{7}	9.1×10^{6}
T3	7.3×10^{9}	4.0×10^{9}	3.6×10^{9}	3.1×10^{9}	1.8×10^{9}	5.2×10^{8}	4.2×10^{8}	3.7×10^{8}	2.5×10^{8}	2.5×10^{8}	2.1×10^{7}	1.1×10^{8}	6.7×10^{7}	3.0×10^{7}
T4	8.1×10^{9}	5.6×10^{9}	4.8×10^{9}	3.9×10^{9}	1.3×10^{9}	1.1×10^{9}	7.3×10^{8}	4.2×10^{8}	3.1×10^{8}	1.4×10^{8}	9.0×10^{7}	3.1×10^{7}	5.6×10^{6}	3.1×10^{6}
T5	10×10^{9}	9.1×10^{9}	8.1×10^{9}	5.8×10^{9}	2.8×10^{9}	9.3×10^{8}	5.2×10^{8}	3.1×10^{8}	1.5×10^{8}	8.0×10^{7}	4.8×10^{7}	1.6×10^{7}	7.9×10^{6}	2.9×10^{6}
IC-7113														
T1	8.3×10^{9}	7.9×10^{9}	4.7×10^{9}	2.65×10^{9}	2.1×10^{9}	8.8×10^{8}	5×10^{8}	4.5×10^{8}	3.7×10^{8}	2.2×10^{8}	1.2×10^{8}	8.6×10^{7}	3.9×10^{7}	1.8×10^{7}
T2	5.1×10^{9}	1.4×10^{9}	9.1×10^{8}	4.2×10^{8}	1.1×10^{8}	6.1×10^{7}	4.8×10^{7}	3.4×10^{7}	2.5×10^{7}	1.3×10^{7}	1.1×10^{7}	9.0×10^{6}	4.8×10^{6}	1.2×10^{6}
T3	4.2×10^{9}	1.4×10^{9}	5.0×10^{8}	1.2×10^{8}	9.0×10^{7}	4.0×10^{7}	2.9×10^{7}	2.3×10^{7}	2.1×10^{7}	1.6×10^{7}	8.8×10^{6}	4.3×10^{6}	1.2×10^{6}	6.1×10^{5}
T4	1.8×10^{9}	9.9×10^{8}	4.0×10^{8}	1.5×10^{8}	1.0×10^{8}	9.8×10^{7}	6.6×10^{7}	4.8×10^{7}	2.3×10^{7}	1.5×10^{7}	8.9×10^{6}	5.8×10^{6}	2.1×10^{6}	4.5×10^{5}
T5	2.8×10^{9}	1.4×10^{9}	8.8×10^{9}	4.6×10^{9}	2.2×10^{9}	6.3×10^{7}	5.4×10^{7}	3.3×10^{7}	2.5×10^{7}	1.9×10^{7}	7.6×10^{6}	3.7×10^{6}	1.2×10^{6}	5.3×10^{5}

TI, peat; T2, talc; T3, talc + starch; T4, charcoal; and T5, charcoal + sugarcane powder

3.4 Work at ICRISAT

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), based at Patancheru, Hyderabad, India, has been using peat-based rhizobial formulation for its mandate crops chickpea, pigeon pea, and groundnut. In order to find an efficient alternative carrier material, a total of six rhizobia (two specific for chickpea, IC-59, IC-76; two specific for pigeon pea, IC-3195, IC-4062; two specific for groundnut, IC-7001, IC-7113) were formulated as five different inoculants using peat, talc, talc amended with starch, charcoal, and charcoal amended with sugarcane powder, and shelf life was evaluated for a period of 13 months (Table 3.2). Among the carrier materials, peat was found to be the best as it holds 10^7 rhizobia for IC-76, IC-3195, IC-4062, IC-7001, and IC-7113 and 10^6 for IC-59 even after 13 months of storage. On the whole, the shelf life maintenance was observed in the order of peat > talc amended with starch > talc > charcoal amended with sugar cane powder > charcoal. The results also suggest that the use of proper additives to the inoculants can tremendously enhance the shelf life of the product.

3.5 Conclusions

From the literature survey, it is observed that legume inoculants gained more attention in developed countries with successful stories like soybean in Brazil, pea and lentil in Canada, and subterranean clover in Australia. In Asia, though there is a considerable interest in rhizobial inoculant development, still many factors such as undisturbed supply of good-quality carrier material, well-developed technology, quality control legislations, well-defined good manufacturing practices, training programs, well-planned field demonstrations, and governmental support for small-scale industries are creating constraints for further development. Unification of all these sectors can lead to the development of a low cost, high shelf life, and highly effective rhizobial inoculants.

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Regulatory Issues in Commercialization of *Bacillus thuringiensis*-Based Biopesticides

4

Estibaliz Sansinenea

Abstract

The utility of biopesticides, as a component of integrated pest management (IPM), has won acceptance over the world. An entomopathogenic organism should be highly specific and effective against the target pest and should be successfully produced. *Bacillus thuringiensis* (Bt) was discovered as a soil bacterium, which fulfills all these requirements and is being used as a biopesticide in agriculture, forestry, and mosquito control. In spite, biopesticides have many advantages as green pesticides and their use has had a slow growth, mainly because the farmers are less confident in selecting biopesticides over the synthetics. However, the global biopesticide market is substantially growing every year. The regulations about the pesticides have many concerns which do not apply to biopesticides, and these issues have been made difficult to introduce them to the market.

Keywords

Biopesticides • *Bacillus thuringiensis* • Integrated pest management • Green pesticide • Entomopathogenic

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4.1 Introduction

4.1.1 Crop Pests and Chemical Pesticides

A pest is an animal or insect that causes problems for people especially by damaging crops. Each species of crop plant is affected by different pest, which varies according to country and region. The natural selection has developed the different mechanisms by which pests affect the crop. These mechanisms are led by the competition between the pest and the plant, between different pest species (e.g., for food and space), and with other members of the ecological community (e.g., with predators or disease) and the abiotic environment. Pest damage can be caused directly (i.e., the plant is eaten by a pest) or it can be caused indirectly, but always there is a reduction in yield or quality due to competition for resources.

When the production of agricultural crops is declining in yield, the farmers often expect a dramatic, magical treatment to make them green and healthy again, so that the productivity increases. Over years, chemical pesticides had been the major contributor against pests and diseases. The use of chemical pesticides was economically a viable strategy because they were cheap and effective. Such pesticides were adopted in the 1940s with the use of dichloro-diphenyl-trichloroethane (DDT), organophosphates, and carbamate pesticides (Nicholson 2007). Their long-term use resulted in insecticide resistance. The contamination of soil and water and the harmful residues of the agricultural products are the most serious problems of the use of chemical pesticides causing tremendous damage to the environment, pest resistance, and lethal effects on nontarget organisms (Abudulai et al. 2001). Chemical fertilizers and pesticides are continuously accumulating in the environment, harming the ecosystem, causing pollution, and inflicting diseases at alarming levels (Gerhardson 2002; Arora et al. 2010). The great use of pesticides has already caused grave damage to health, ecosystems, and groundwater due to its survival in plants for a long time as a residual. They also enter in the food chain and are found in meat and dairy products and remain as residue in the soil and ecosystem for long periods of time (Bisen et al. 2015; Keswani et al. 2013).

4.1.2 Bt-Based Biopesticides: A Green Solution to Chemical Pesticides

For the reasons given above, many farmers and growers over the world are trying to reduce the amounts of conventional chemical pesticides used. Integrated pest management (IPM) is a program that combines different crop protection practices with careful monitoring of pests and their natural enemies. Biopesticides are a particular group used in IPM and offer the possibility to create a new generation of sustainable agriculture products. In very general terms, according to the US EPA, biopesticides, also known as biological pesticides, are derived from natural materials such as animals, plants, bacteria, and certain minerals (US Environmental Protection Agency Pesticides 2014). Typically, biopesticides have unique modes of action and are

considered reduced risk pesticides. Biopesticides are not used to eradicate pests but to control pests with the advantage of a major selectivity and nontarget biological safety. Biopesticides can be classified into three major classes: biochemical, plant-incorporated protectants, and microbial pesticides, which are also known as BCAs. Among the last group, there can be found microbial pesticides that function in multiple ways. The most widely used microbial pesticides are based on *B. thuringiensis* (Bt), accounting for approximately 90 % of the biopesticide market in the USA (Chattopadhyay et al. 2004).

The most widely used microbial biopesticide is the insect pathogenic bacterium *Bacillus thuringiensis* (Bt), which produces an endotoxin protein called Cry during sporulation that is able to lead to lysis of the gut cells when consumed by susceptible insects (Gill et al. 1992). The δ-endotoxin is very specific and can cause death within 48 h (George and Crickmore 2012). It is safe to people, beneficial organisms, and the environment. Bt biopesticides consist of a mix containing bacterial spores and δ-endotoxin crystals mass-produced in fermentation tanks and formulated as a sprayable product. Bt sprays are a great option for pest management on several crops where resistance to synthetic chemical insecticides is a problem (Abdullah 2012). Bt sprays have also been used on broad-acre crops such as maize, soya bean, and cotton. However, the use of Bt microbial biopesticide formulations has been rather scarce due to the problems of narrow host range, low persistence on plants, and inability of foliar application to reach the insects feeding inside the plants, notwithstanding several biotechnological approaches for the development of improved Bt biopesticides (Kaur 2007).

The problems of field application of Bt biopesticides have been overcome by Bt transgenic crops. The total acreage of transgenic crops has been steadily increasing with commercial cultivation of transgenic crops on 140 million hectares in 2010 (James 2010). The most widely grown Bt crop is cotton (*Gossypium hirsutum* L.), accounting for 64% of global cotton area devoted to Bt crops, followed by corn (*Zea mays* L.) accounting for 29% of global corn area. Bt cotton has been planted on an increasingly large scale in India and China (Liu 2009; Chaudhary and Gaur 2010). The use of Bt crops has resulted in increased yields and significant reductions of insecticide application, thus generating economic and environmental improvements (Shelton et al. 2008; Brookes and Barfoot 2008; Carpenter 2010). However, while there are undoubted advantages of deployment of Bt transgenic crops for effective insect pest control, certain concerns have been raised about the environmental safety of Bt transgenic crops.

4.2 Efficacy and Safety of Bt-Based Biopesticides

Generally speaking, there are different benefits to use biopesticides in comparison with conventional chemical pesticides. In sum, biopesticides tend to be less toxic, more quickly biodegradable, and more targeted to the specific pest (Leahy et al. 2014). Biopesticides are not designed to eradicate a target pest but to control a pest population to a manageable level (Lewis et al. 1997). These points achieve benefits

to humans and ecosystems and reduce concerns for development of pest resistance to existing control tools.

The success of Bt-based biopesticide production depends on high-quality and high-efficiency formulation processes, which must be safe and effective; the products must be easy to use and should have a long shelf life. The spore-crystal complex is the active ingredient in commercial formulations, which is more effective to use and cheaper to obtain than the crystals alone and must be helped by suitable inert substance that can function to protect the spore-crystal complex or to increase availability to insects. Bt sprays are used sporadically and typically over small areas over cotton, fruit, and vegetable crops. However, the use of Bt spray as an insecticide has several disadvantages: (1) Bt spray cannot be applied uniformly to all parts of the plant, (2) it cannot be applied inside plant tissues, and (3) Bt is susceptible to rapid degradation by UV light and removal by water runoff. Therefore, multiple applications are required to provide extended pest protection. New Bt formulations have consistently come to vegetable markets over the last number of years (Cerón 2001). The persistence of Bt spores in different environments has been reasonably well studied (Petras and Casida 1985). Bt spores can survive for several years after spray applications, although rapid declines in population and toxicity have been noted (Addison 1993). The bioassays for Bt products have had a standardization procedure. Before 1970, the standardization procedure was carried out through the use of spore counts. However, there was no relationship between the number of spores in a preparation and its insect killing power. Because of this actually standardization procedure is carried out using insect bioassay. Insect bioassay of Bt products is expensive and time consuming and takes a relatively long time (4–7 days) to furnish information on the potency of the material. Due to these reasons, insect bioassays have been replaced with chemical assays. There are several things to take into consideration using chemical assays. The killing capacity of the product toward target pest insects is the required information. The killing capacity of the product is determined by both the quality and quantity of crystal toxin present, and in some cases the crystal toxin requires the presence of spores to have toxicity against pest insects. Chemical methods only measure the quantity of toxin present, but not measure the quality of toxin present nor the presence, number, or viability of Bt spores. Because of this, many laboratories still check their Bt preparations by insect bioassay (Beegle and Yamamoto 1992).

The efficacy of Bt microbials applied to the surface of leaves is limited by the fact that the formulation can be washed off by rain and the Cry proteins are inactivated by sunlight within a few days of application (Federici and Siegel 2008). To solve the problem of the damage of UV irradiation to *B. thuringiensis*, some chemical screens have been used. However, these chemical screens have some negative impacts on the environment. In contrast, melanin is a natural pigment that is easily biodegradable in the nature and can absorb radiation; therefore it is a perfect photo protective agent, which has been used to protect Bt formulations from UV light (Sansinenea and Ortiz 2015; Sansinenea et al. 2015).

With the development of biotechnology, it has been possible to introduce the genes coding for Cry proteins into plants so that Cry proteins are expressed in the

plant and are produced throughout the growing season to provide protection against insect pests. The efficacy of Bt is dependent on its narrow spectrum of toxicity too. Most of the Bt products in agriculture are targeted against lepidopteran insects. Also, since it is only active when ingested by the specific target pests, topically applied Bt is not effective against insects that normally bore into plant tissues such as stem and fruit borers (i.e., *Ostrinia nubilalis* and *Helicoverpa zea*) and leaf miners (i.e., *Tuta absoluta* and *Phyllocnistis citrella*). However, these limitations could be overcome; *O. nubilalis* is effectively controlled by genetically modified corn that expresses Bt toxin in its tissues (reviewed in (Sanahuja et al. 2011; Meissle et al. 2011)), while the addition of surfactant appears to increase the toxicity of topically applied Bt against leaf miner *P. citrella* by increasing Bt penetration into the mines (Shapiro et al. 1998).

4.3 Commercialization and Market of Biopesticides

A registered biochemical or microbial pesticide contains one or more active ingredients which are primarily responsible for the pesticide claims. But the final formulation contains one to dozens of other ingredients called "inerts." This term can be confusing, as it implies that these components do not have a particular function but they are required to make an effective product. Sometimes inert ingredients can have serious potential health and ecosystem impacts. In the case of biopesticides, this is problematic; a company can combine in one formulation highly targeted, benign active ingredients that include a dangerous inert ingredient.

The costs of market entry of biopesticides are proportionately higher for some small companies, in contrast to large companies, because there are some details such as large-scale productions of broadly applicable, easily applied products and those that are niche applications and pest and life cycle specific that need paying attention to.

China has been probably the biggest user of Bt microbial pesticides where, over the last few decades, tens of thousands of tons of various Bt microbial formulations have been topically applied on agricultural food crops (rice, vegetables, maize), in forests, and to potable water to control mosquitoes and other larval insects that are vectors of human disease (WHO/ICPS 1999; Ziwen 2010). According to recent data, there were at least 180 registered Bt microbial products in the USA (EPA 1998) and over 120 microbial products in the EU. There are reported to be nearly 276 Bt microbials registered in China (Huang et al. 2007).

Worldwide, approximately 1400 biopesticide products were being sold (Marrone 2007). Table 4.1 depicts a comprehensive list of commercially available Bt-based biopesticides in the markets around the globe. These products are commercially successful and available in different formulations such as liquid concentrates, wettable powders, and ready-to-use dusts and granules.

The production of Bt always remained on priority in biopesticide industry, and currently it is the main bacterium being used in agricultural pest control (Brar et al. 2006; Ali et al. 2008). Its firm position in biopesticide industry is indicated by the

Table 4.1 Commercially available Bt-based biopesticides in the global market

Category of biopesticide	Products common name or trade name	Targets
The USA		
B. thuringiensis subsp. *aizawai* NB200	Florbac	Moth larvae
B. thuringiensis subsp. *israelensis*	BMP	Mosquito and black flies
B. thuringiensis subsp. *israelensis* EG2215	Gnatrol	Mosquito, flies
	Aquabac	
B. thuringiensis subsp. *aizawai* delta-endotoxin in killed *P. fluorescens*	M-Trak	Colorado potato beetle
B. thuringiensis subsp. *aizawai* GC-91	Agree WG	Plutella
B. thuringiensis subsp. *kurstaki*	Thuricide Forestry	Lepidopteran larvae
	Wilbur-Ellis BT 320	
	Dust	
	Dipel	
	Deliver	
	Biobit HP	
	Foray	
	Javelin WG	
	Green Light	
	Hi-Yield Worm Spray	
	Ferti-Lome	
	Bonide	
	Britz BT	
	Worm Whipper	
	Security Dipel Dust	
B. thuringiensis subsp. *kurstaki* BMP 123	BMP123	Lepidopteran larvae
B. thuringiensis subsp. *kurstaki* EG2348	Condor	Lepidopteran larvae
B. thuringiensis subsp. *tenebrionis*	Novodor	Colorado potato beetle
B. thuringiensis subsp. *kurstaki* EG7826	Lepinox WDG	Lepidopteran larvae
Europe		
B. thuringiensis subsp. *aizawai* GC-91	Turex	Lepidopteran pests
B. thuringiensis subsp. *israelensis* AM65	VectoBac	Sciarids
B. thuringiensis subsp. *kurstaki* HD-1	Dipel WP	Lepidopteran pests

(continued)

Table 4.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
B. thuringiensis subsp. *kurstaki* ABTS	Batik	Lepidopteran pests
351, PB 54, SA 11, SA12, and EG 2348	Delfin	
B. thuringiensis subsp. *kurstaki*	BMP 123	Lepidopteran pests
BMP 123	Prolong	
B. thuringiensis subsp. *tenebrionis*	Novodor	Coleopteran pests
NB 176		
China		
B. thuringiensis subsp. *aizawai*	Trade name not available	Lepidopteran pests
B. thuringiensis subsp. *israelensis*	Trade name not available	Lepidopteran pests
B. thuringiensis subsp. *kurstaki*	Trade name not available	Lepidopteran pests
Japan		
B. thuringiensis kurstaki	Toarowaa Esmark Guardjet, Dipol, Tuneup Fivestar BioMax DF	Lepidopteran larvae
B. thuringiensis aizawai	Quark Xen Tari Florbac Sabrina	Lepidopteran larvae
B. thuringiensis aizawai + *kurstaki*	Bacilex	Lepidopteran larvae
B. thuringiensis japonensis	Bui Hunter	Cockchafers and white grubs
India		
B. thuringiensis subsp. *israelensis*	Tacibio, Technar	Lepidopteran pests
B. thuringiensis subsp. *kurstaki*	Bio-Dart	Lepidopteran pests
	Biolep	
	Halt	
	Taciobio-Btk	
Australia		
B. thuringiensis subsp. a*izawai*	Agree, Bacchus, XenTari	Lepidopteran larvae
B. thuringiensis subsp. *israelensis*	Aquabac, BTI, Teknar, Vectobac	Mosquito larvae
B. thuringiensis subsp. *kurstaki*	Biocrystal, Caterpillar, Killer, DiPel, Costar, Delfin, Full-Bac WDG	Lepidopteran larvae
Africa		
B. thuringiensis subspp. *aizawai* and *kurstaki*	Agree	Lepidopteran larvae
B. thuringiensis subsp. *israelensis*	VectoBac	Mosquito
B. thuringiensis subsp. *kurstaki*	DiPel	Lepidopteran larvae
	Rokur	
	Thuricide	
B. thuringiensis subsp. *kurstaki*	H7 Florbac WG	Lepidopteran larvae

Source: Kunimi (2007) and Kabaluk et al. (2010)

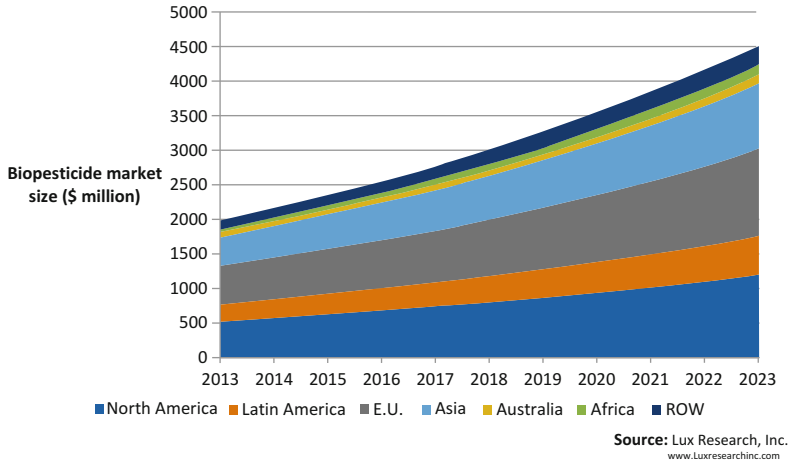

Fig. 4.1 The biopesticide market is approximately $3 billion today and will rise above $4.5 billion by 2023

fact that more than 53 % of the world biopesticide market is occupied by about 200 Bt-based products (CABI 2010), and almost 50 % of this is consumed by America particularly in the USA and Canada (Guerra et al. 2001).

Today, biopesticides make up a small fraction of the total global crop protection market at approximately $3 billion in value worldwide (Olson 2015) (See Fig. 4.1). The growth would be regional, with Europe and Latin America projected to grow most quickly in the coming 3 years, driven by tightening regulatory restrictions and rapidly emerging insect resistance, respectively. Africa is poised for significant growth, but in a more extended time frame. North America, which already accounts for a large proportion of the market, will continue to grow at a slower rate than Europe.

Since 2012, multiple acquisitions, licensing agreements, and partnerships with values well into the hundreds of millions of dollars show the depth and breadth of investment large companies are making in biopesticide development. The bigger companies of the market such as Bayer, Syngenta, BASF, DuPont, Dow AgroSciences, and Monsanto account for 70 % of the world pesticide sales market.

4.4 Regulations About Biopesticides

The regulation of biopesticides takes place within a regulatory system of synthetic (chemical) pesticides. Hence, the system has a number of steps that made it tedious to the registration of biopesticides, and a number of adjustments have been made to the system to facilitate their registration. The pesticide regulation system seems to act as barriers to the further development and commercialization of alternative control methods. One of the objections made by the opponents to pesticides is "how

pesticides are spread directly into nature, in contrast of industrial chemicals" (Blok et al. 2006). Taking into consideration that biopesticides come from nature, these objections do not apply in the same way, but this has not been fully recognized by the regulatory system.

In the process of pesticide development, field testing is often necessary to evaluate the efficacy of a pesticide. Briefly, when the size of the outdoor test acreage is greater than a cumulative 10 acres of land or 1 surface acre of water, an experimental use permit (EUP) is required. Any food or feed crops involved in or affected by the tests must be destroyed or consumed only by experimental animals unless a tolerance has been established. These acreage limitations are applicable only for outdoor terrestrial and aquatic uses. For those pesticides being tested on sites for which acreage is not relevant (e.g., tree stumps, rodent control, structural treatments, or bird repellents), the determination of the need for an EUP is made on a case-by-case basis.

An EUP is of limited duration and requires that the test be carried out under controlled conditions. For small-scale field tests of genetically modified microbial pesticides or nonindigenous microbial pesticides that the USDA has not previously acted upon, applicants must submit a notification to the EPA for the determination of whether an experimental use permit is necessary, even if the testing is on less than 10 acres.

Registration of biopesticide is the main hurdle in the development, and most of the time, registration is much more expensive than the production. Registration is not only expensive but also time consuming (Ehlers 2006). The main problem is that biopesticides contain active cells (live organisms), and these live forms are treated like pathogens by the government agencies. Another issue is regarding the import and export of biopesticides; again, it should be pointed that export and import of chemical pesticide are much easier (as no one doubts on its integrity) in comparison to the use of biopesticides.

The assessment of risks is important to approve or register new biological pesticides and whether to renew the registration of old ones. Registration requires collation of data and preparation of dossier for submission to a national regulatory authority. By these efforts, governments can speed up the process of approving safer new pesticides and stopping the use of riskier ones.

Before a pesticide can be marketed and used in the USA, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requires that the EPA evaluates the proposed pesticide to certificate that its use will not have unreasonable risks of harm to human health and the environment. This involves an extensive review of health and safety information. Pesticide registration is also the process through which the EPA examines some points such as the ingredients of a pesticide; the site or crop on which it is to be used; the amount, frequency, and timing of its use; and storage and disposal instructions. A pesticide cannot legally be used, sold, or distributed if it has not been registered with the EPA's Office of Pesticide Programs.

As biopesticides are usually less toxic than chemical pesticides, biopesticide registrations may require a significantly reduced data set compared to conventional

registrations. Additionally, there are reduced associated timelines and fees to help expedite registration processes.

The manufacturer must submit data on a broad range of toxicological end points. The Office of Pesticide Programs reviews toxicity and exposure information for each active ingredient and dictates approved use conditions. The US EPA provides a fast-track review and registration for biopesticides by combining lower data requirements with high review priority.

In spite of the review process for biopesticides being more efficient in bringing them to the market, the main focus of the EPA pesticide registration process is on human health and safety. On the one hand, speed to market is an economically important goal, but on the other hand, the environment and health data gaps must be attended since otherwise it will lead to serious problems followed by backlash against them.

4.5 Conclusions

The use and situation of biopesticides still remain in dilemma. Farmers find themselves confused and less confident in selecting biopesticides over the synthetics. Despite the fact that presently biopesticides are being used everywhere in the world, it is also known that developed countries seem to be ahead in their wider application (Chandler et al. 2011).

Also countries like India are vastly dependent upon agriculture for not only feeding their populations but also for the economy which depends majorly on this sector. However, most of the challenges faced for the upliftment of biopesticides are fundamental and cosmopolitan. These include the efficacy of the microbial activity, survival of microorganisms, delivery systems, determining host range, avoiding injury to nontarget organisms, consistency, performance in field conditions, economics, government regulations, and confidence among the end users. Gelernter (2007) has described the future of biocontrol in Asia, and according to him unreasonable expectations for performance, inappropriate regulatory guidelines, lack of documentation on the uptake of microbial control strategies, difficulties in implementing local production schemes, and inhibition of scientific exchange are the main hurdles in establishment of biocontrol.

In spite of all these limitations, biopesticides are gradually becoming popular around the world. While we cannot predict the growth of the biopesticide market to the dollar, we are confident in our assessment that the industry will continue to grow in the future. The applications of biopesticides have a bright future. However, it is necessary to control the use of biopesticides, because some kinds of products might result in environment pollution or be harmful to the natural enemies.

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Beauveria bassiana as Biocontrol Agent: Formulation and Commercialization for Pest Management

5

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Abstract

Beauveria bassiana is the most widely used biocontrol agent against many major arthropod pests. This ascomycetal fungus is able to produce infection structures and synthesize a cocktail of proteins, enzymes, organic acids, and bioactive secondary metabolites, which are responsible for the entomopathogenic activity and virulence. For commercial purposes, *B. bassiana* is usually formulated using conidia with different stabilizing agents. Various types of formulation include bait/solid, encapsulation, and emulsion. Commercialization and marketing strategies, including alternative marketing channels, such as earthworm compost and compost, along with the legal framework are addressed in this chapter.

Keywords

Beauveria bassiana • Biocontrol • Entomopathogen • Pest management

5.1 *Beauveria bassiana*: A Fungal Biocontrol Agent

There is an increasing interest in the development of alternatives to replace or complement conventional pesticide usage for crop protection. The use of biological control agents, particularly fungal species, represents a benign, sustainable, and eco-friendly strategy and has been proven to be effective against different pests. One of these fungal biocontrol agents is *B. bassiana*, which is the most widely used

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entomopathogenic fungal species available commercially in different formulations against many major arthropod pests in agricultural, urban, forest, livestock, and aquatic environments (Faria and Wraight 2007; Goettel et al. 2010; Keswani et al. 2013; Singh et al. 2014).

B. bassiana (Balsamo) Vuillemin is a ubiquitous soilborne anamorphic fungus of the Clavicipitaceae family, which completes the asexual life cycle (based on the formation of conidia and germination) as saprophyte in soil and on other organic materials, although it has also been reported as an endophyte in several plants (Vega et al. 2008). This facultative necrotrophic entomopathogenic ascomycete behaves as a parasite of insects and arachnids (Rehner 2005; Rehner et al. 2011), which seems to be crucial for the sexual life cycle, since the teleomorph stage (*Cordyceps bassiana*) has been only sparsely reported on cadavers of arthropods in eastern Asia (Li et al. 2001; Huang et al. 2002; Sung et al. 2006).

The entomopathogenic activity requires the production of infection structures (appressoria), metabolites, proteins, and enzymes, which will allow *B. bassiana* conidia to adhere to the host arthropod, penetrate the cuticle, proliferate in the hemocoel as blastospores (hyphal bodies capable of evading the host immune system (Lewis et al. 2009)), and ultimately kill the host. Then *B. bassiana* hyphae reemerge, cover the cadaver, and form new conidia, thus completing the parasitic life cycle (Toledo et al. 2010; Ortiz-Urquiza et al. 2010, 2015; Ortiz-Urquiza and Keyhani 2013).

5.2 Bioactive Metabolites, Proteins, and Enzymes Produced by *B. bassiana*

Entomopathogenic fungi are capable of implementing different mechanisms aimed to parasitize arthropods. These mechanisms include the production of proteins, enzymes, organic acids, and bioactive secondary metabolites.

5.2.1 Hydrolytic Enzymes, Proteins, and Organic Acids

Although it has been suggested that hydrolytic enzymes represent the primary infection mechanism that allows for penetration of fungal hyphae through the arthropod cuticle (Ortiz-Urquiza and Keyhani 2013), adhesion to and interaction with the epicuticular layer of the host must occur first. In *B. bassiana*, at least two hydrophobins (Hyd1 and Hyd2) are in charge of fungal spore coat rodlet layer assembly, thus contributing to cell surface hydrophobicity, adhesion to hydrophobic surfaces, and virulence (Cho et al. 2007; Zhang et al. 2011). Assimilation of the lipids, hydrocarbons, proteins, and other compounds included in the cuticular layer requires the synthesis of different fungal enzymes, such as cytochrome P450, catalases, esterases, long-chain alcohols, and aldehyde dehydrogenases (Pedrini et al. 2006, 2010, 2013; Ortiz-Urquiza and Keyhani, 2013). Other hydrolytic enzymes related to virulence are known to be secreted by *B. bassiana* and include proteases,

glycosidases, lipases, and chitinases, which promote germination, fungal growth, and subsequent penetration inside the host (St Leger et al. 1986, 1997; Fan et al. 2007; Zhang et al. 2008; Fang et al. 2009). *B. bassiana* also produces a bioactive protein named bassiacridin. This insecticidal 60-kD protein has β-glucosidase, β-galactosidase, and N-acetylglucosaminidase activities (Quesada-Moraga and Vey 2004).

In addition to this hydrolytic and detoxifying enzyme cocktail, the production of organic acids (mainly oxalic acid) also contributes to *B. bassiana* virulence (Kirkland et al. 2005), since oxalic acid is able to weaken the integrity of insect cuticle (Bidochka and Khachatourians 1991).

5.2.2 Bioactive Secondary Metabolites

Not only compounds from primary metabolism participate in the parasitization process. Low molecular weight bioactive secondary metabolites produced in vitro and in vivo by *B. bassiana* play an important role as (a) toxins that cause arthropod's death, (b) immunomodulators that aid the fungus to evade the host defense system, (c) antimicrobials against competing microorganisms, and (d) defense molecules against mycophagous organisms (Charnley 2003). *B. bassiana* has an enormous potential to produce secondary metabolites, since 13 non-ribosomal peptide synthetases (NRPS), 12 polyketide synthases (PKS), 7 NRPS-like, 1 PKS-like, 3 hybrid NRPS–PKS, and 12 genes related to FAS/terpene/steroid biosynthesis are encoded within its genome (Xiao et al. 2012). The known secondary metabolites produced by this entomopathogenic fungus include cyclic peptides, such as beauvericin, bassianolide, and beauverolides, and polyketide-derived pigments, such as oosporein, tenellin, and bassianin, but only those genes involved in the biosynthesis of beauvericin, bassianolide, tenellin, and oosporein have been functionally verified (Roberts 1981; Strasser et al. 2000a, b; Vey et al. 2001; Molnar et al. 2010; Xu et al. 2008, 2009; Eley et al. 2007; Halo et al. 2008; Feng et al. 2015).

5.2.2.1 Cyclic Peptides

Beauvericin is probably the most studied cyclic peptide compound produced by *Beauveria* spp. This cyclooligomer hexadepsipeptide is an acyclic trimer of the dipeptidol monomer D-hydroxyisovaleric acid– N-methyl-L-phenylalanine and is also synthesized by *Paecilomyces* and a number of *Fusarium* spp. (Wang and Xu 2012; Covarelli et al. 2015). Beauvericin possesses antiviral and broad-spectrum antibacterial activities and is able to potentiate the antifungal properties of other fungicides (Shin et al. 2009; Wang and Xu 2012; Fukuda et al. 2004a,b; Zhang et al. 2007). Beauvericin is a strong insecticidal molecule (Hamill et al., 1969), but the exact mechanism of action remains to be elucidated (Wang and Xu 2012). In addition, this hexadepsipeptide has cytotoxic and proapoptotic activities in several human cell lines, including leukemia cells (Jow et al. 2004, Calo et al. 2004; Lin et al. 2005; Wang and Xu 2012). Beauvericin seems to act as an ionophore, forming cation-selective channels and increasing intracellular Ca^{2+} concentrations

(Wu et al. 2002; Kouti et al. 2003) which have been suggested to trigger calcium-sensitive cell apoptotic pathways (Jow et al. 2004; Wang and Xu 2012). Other authors have reported that the apoptotic effect of beauvericin is mediated by Bc1-2 proteins, cytochrome c, and caspase 3 (Lin et al. 2005) and by the activation of the JNK signaling pathway, inhibition of both TNFα-induced NF-kB activation, and phosphorylation of ERK (p44/p42) (Wätjen et al. 2014).

Bassianolide is another cyclooligomer that might also be important during insect pathogenesis (Xu et al. 2008, 2009), since this molecule, together with beauvericin, has been isolated from extracts of *Bombycis corpus* inoculated by *B. bassiana* (Kwon et al. 2000). This cyclic octodepsipeptide tetrameric ester of the dipeptidol monomer D-hydroxyisovaleric acid–N-methyl-L-leucine is produced by *B. bassiana* and *Lecanicillium* sp. (*Verticillium lecanii*) (Suzuki et al. 1977). This compound exhibits antibacterial (against some *M. tuberculosis*), antimalarial, and cytotoxic (against several tumor cell lines) activities (Kwon et al. 2000; Jirakkakul et al. 2008). Bassianolide insecticidal properties are due to its ability to inhibit acetylcholine-induced smooth muscle contraction (Nakajyo et al. 1983), thus inducing atony and toxicity to different insect larvae (Suzuki et al. 1977; Champlin and Grula 1979).

Other cyclic peptides include the beauverolides (beauveriolide or beauverilide) and lipophilic and neutral cyclotetradepsipeptides that vary in amino acid composition and contain linear and branched β-hydroxy acid residues of variable length (e.g., beauverolide M is made up of Val–Ala–Leu and contains 3-hydroxy-4-methyloctanoic acid, whereas beauveriolide L is made up of Phe–Ala–Ile and contains 3-hydroxy-4-methyldecanoic acid). These metabolites are produced by entomopathogenic species of the genera *Beauveria* (including *B. bassiana*) and *Paecilomyces* (Elsworth and Grove 1977; Jegorov et al. 1994). They seem not to have bactericidal, fungicidal, or direct insecticidal effects, although they apparently have an immunomodulatory role in insects (Jegorov et al. 1990; Mochizuki et al. 1993; Vilcinskas et al. 1999).

5.2.2.2 Polyketide-Derived Pigments

Oosporein is a di-symmetric cyclohexadienedione (dibenzoquinone) whose biosynthesis involves a PKS (Feng et al. 2015). This red pigment is synthesized by *B. bassiana* and other fungi (el-Basyouni and Vining 1966; Strasser et al. 2000a, b; Mao et al. 2010; He et al. 2012; Ramesha et al. 2015). It can naturally occur in food and feed and contaminate many important crops, this mycotoxin being capable of producing adverse acute and chronic effects in animal health (Manning and Wyatt 1984; Cole et al. 1974; Pegram and Wyatt 1981; Brown et al. 1987). Oosporein exhibits broad-spectrum antimicrobial and antifungal activities (Brewer et al. 1984; Strasser and Abendstein 2000; Alurappa et al. 2014; Toshinori et al. 2004; Mao et al. 2010). Antitumor, antioxidant, and cytotoxic properties have also been reported for oosporein (Mao et al. 2010; Alurappa et al. 2014; Ramesha et al. 2015). The induction of elevated levels of reactive oxygen species (ROS) has been recently proposed as the mechanism of toxicity of this pigment (Ramesha et al. 2015).

Tenellin and bassianin are yellow pigments with a 2-pyridone ring that have been isolated from *Beauveria* species (Eley et al. 2007; McInnes et al. 1974). Bassianin differs from tenellin by one chain extension in the ketide moiety. These two compounds, in addition to oosporein, are able to inhibit erythrocyte membrane APTase activity, which is likely a consequence of the ability of these pigments to promote varying degrees of cell lysis by means of membrane disruption (Jeffs and Khachatourians 1997). Although tenellin is not involved in the pathogenesis of *B. bassiana* against honeycomb moth (*Galleria mellonella*), it can prevent iron-generated reactive oxygen species toxicity in *B. bassiana* (Eley et al. 2007; Jirakkakul et al. 2015).

5.3 Formulations of *B. bassiana* for Pest Biocontrol

Some desirable characteristics, such as ease of preparation and application, stability, low cost, and abundant viable propagule, are pursued in order to obtain an appropriate pest biocontrol formulation. Entomopathogenic fungi are usually included in the form of conidia to facilitate the application in formulations, which, in addition, need stabilizing agents for proper storage and enhancement of activity.

The three main formulations that include *B. bassiana* are bait/solid (usually tea waste based), encapsulation, and emulsion.

5.3.1 Bait/Solid Formulation

Bait formulation consists of *B. bassiana* conidia as active ingredient, mixed with food or another attractive substance. In the case of *Beauveria* formulations, the abundantly available tea waste is one of the most common ingredients used for the production of these baits. It provides an economically viable option with a simple preparation methodology, and the technology can be easily replicated at the end user level (Mishra et al. 2013).

In spite of all the advantages regarding low cost, simple methodology, and ease of transport (facilitating mass applicability), the application and shelf life of bait formulations present several disadvantages.

In addition to the difficulties to get an even distribution during application of bait formulations, the major problem is the storage ability and the short shelf life, which is limited to 2–3 months (Mishra et al. 2013). Probably, this handicap makes the commercialization of bait formulations more difficult, since the short shelf life limits the functional area of use and confines bait formulations to local production and utilization.

Also, under controlled laboratory conditions, some of the wettable powder bait formulations of *B. bassiana* have finally resulted in slightly greater mortality of conidia than the same composition formulated as an emulsifiable suspension (Parker et al. 2015).

Bait formulations of *B. bassiana* are at the risk of killing potential beneficial nontarget organisms. In addition, they can also serve as food supply for other pests after removal of fungal conidia, thus generating an unwanted effect (Bukhari et al. 2011).

Some more complex solid formulations, such us carrier-based powder formulation (CBPF), incorporating powder, glycerine, and gum, have been also tested for efficacy and viability, showing intermediate values in comparison with naturally more stable-based liquid formulations (Ritu et al. 2012).

5.3.2 Encapsulation

Encapsulated formulations of *B. bassiana* protect fungal conidia from adverse environmental conditions and usually increase shelf life and bioefficacy. The use of additives (skimmed milk powder, polyvinyl pyrrolidone K-90, and glucose) improves handling of formulation and allows a better distribution of *B. bassiana* conidia, although the encapsulation technique exerts a negative effect on conidial viability (Mishra et al. 2013).

The main effects of using additives in encapsulated formulations have been described on:

(a) Conidial viability: Encapsulated conidia-containing additives (mainly glucose and sucrose) showed comparatively higher conidial viability, suppressing the abovementioned detrimental effect of encapsulation process. This has been attributed to the protective effect of these sugars during freeze drying. Addition of sugar in the encapsulation process becomes highly relevant at field application stage, since sugars seem to improve the viability of encapsulated conidia by creating a niche osmotic protective environment (Mishra et al. 2013).
(b) Germination kinetics: Addition of glucose and sucrose to encapsulation formulations increases growing trend (probably due to a nutritive effect), while germination kinetics are negatively affected when mannitol is used as added sugar (Liu et al. 2015).

5.3.3 Emulsion

The emulsion formulation of entomopathogenic fungi with vegetable oil seems to be a very suitable option. Emulsions are easy to apply and protect fungal conidia from UV radiation, thus increasing their efficacy and pathogenicity against insect pests by promoting conidial adhesion on the insect's cuticle.

Emulsion formulations are usually prepared with vegetable oils, most commonly soybean, rapeseed, sunflower, olive, tile, and linseed, but also almond, gingelly, coconut, castor oil, mustard, and eucalyptus oil (Sankar-Ummidi and Vadlamani 2014).

Some synthetic oils have been also evaluated as ingredients for emulsion formulations, since they seemed to be more easily mixed and later applied to a water surface, thereby improving the persistence of fungal spores after their application in fields (Bukhari et al. 2011).

Usually, these emulsions are prepared in an oil-in-water formulation by adding a surfactant (mainly Tween 20), mixing the oil phase with the aqueous phase containing the spore suspension. The aqueous phase with the conidial suspension is mixed with sterilized oil at the effective concentration, and other optional ingredients such as Triton X-100 (as nonionic surfactant), Na_2CO_3 (as stabilizer), and silicon (as antifoaming agent) can be added. Finally, mixtures of these two phases are homogenized to get a stable formulation (Yacoub and Batta 2016).

The compatibility of most of these vegetable oils (and synthetic oils) has been successfully evaluated on conidia from *B. bassiana* in terms of effectiveness, taking into consideration parameters such as germination rate, vegetative growth, and conidiogenesis (Sankar-Ummidi and Vadlamani 2014; Gomes et al. 2015).

Different oil emulsion formulations of *B. bassiana* have shown a variable reduction in spore germination, vegetative growth, and conidia production. Variation in conidial germination due to different oils has been attributed to some qualitative (and quantitative) composition of fatty acids, since different proportions of unsaturated fatty acids contained in the oils, such as linoleic acid and oleic acids, have antifungal properties. In this regard, the linseed oil emulsion formulation has shown a maximum conidial germination rate, unlike other emulsion formulations containing even very low concentrations (1 %) of other oils (e.g., mustard and eucalyptus), which have been reported as toxic for *B. bassiana*. In the case of eucalyptus oil, the toxic effect has been attributed to its active ingredient citronellal (Sankar-Ummidi and Vadlamani 2014).

Conidial germination in some oil emulsions (e.g., linseed) has been evaluated under storage conditions (standard temperature of 30 ± 2 °C) for 12 months, showing a significant decrease in conidial viability (deterioration in mycelium and undetectable fungal conidia). Lower storage temperature is being evaluated to assure further longevity of formulated conidia (Mishra et al. 2013).

In the case of insect pests, entomopathogenic fungi formulated in oil emulsions show a clear increase in virulence, likely due to better ability of the oiled conidia to adhere the lipid layer of insect cuticle through hydrophobic interactions, later facilitating germination and progression of the infection process (Ment et al. 2010). Addition of some carriers, such as the clay bentonite, to oil-based liquid formulations has been reported to improve the efficacy of infection of *B. bassiana* (Ritu et al. 2012). The effectiveness of *Beauveria* emulsion formulations increases when more complex pheromone trapping systems–oil emulsions are combined, since part of the individuals are infected with a heavy load of spores directly by contact before they leave the trap, thus providing an excellent and highly effective indirect infection way for other non-trapped individuals, mainly through their mating behavior (Hajjar et al. 2015).

Emulsions are excellent spray carriers that increase the probability of direct contact between fungal conidia and pests. Oils in the emulsion are reported to prevent evaporation in field and increase in situ conidial retention. These properties represent further advantages of oiled emulsions of *Beauveria*, making this formulation an excellent choice for the biocontrol of habitats difficult to penetrate (Mishra et al. 2013).

5.4 Commercialization and Administration of *B. bassiana*

In an increasingly globalized world, the core facilities for fermentation and production of *B. bassiana* are thousands of kilometers away from the market place. That is, the first step in the distribution chain is the export–import process.

5.4.1 Import–Export Process

The Harmonized System 6-digit number (HS code) is given to each product capable of passing through customs. It is an international system respected by the vast majority of countries. The fundamental problem concerning international trade of *B. bassiana* is the lack of a specific item in the HS for these products. This creates difficulties in custom processes, as each country has a specific interpretation of the code, thus requiring arbitrary documentation and inspections.

In general, the 3808 91code is recommended for this product (although it should be contrasted with the local custom institution) because this tariff item includes those products with insecticidal effect improperly described elsewhere. Also, the 3808 91code itself expressly refers to biopesticide products based on *Bacillus thuringiensis*, a similar product in terms of effects and nature. The usual documentation required in this process includes certificate of origin, supplier's manufacturing license, health certificate, letters of use, and destination, among others.

5.4.2 Product Application

Regardless of the specific formulation of *B. bassiana*, application of these products is recommended to proceed through foliar sprays, ensuring that leaves are properly inoculated. General recommendations include:

(a) Powder formulations: Four kilograms shall be mixed with 20 L of water. Stir and wait until the carrier (usually talc) settles at the bottom of the container. Then, take the liquid and mix with 500 L of water to apply it through the drip irrigation system or through the foliar spray system.
(b) Liquid formulations: Directly mix the selected dosage (see below) with 500 L of unchlorinated water.

5.4.3 Dosage

Commercial dosages greatly vary depending on the type of formulation, but in general, assuming a CFU of 10^9 in liquid formulations and 10^8 in powder formulations, 3 L/Ha and 4 kg/Ha, respectively, should be applied to control pests. In the case of severe infestation, apply every 2 weeks.

5.5 Distribution Channels and Marketing of *B. bassiana*

Like for every agricultural product, introduction of the biocontrol product in the market is as important as the development of an innovative and effective formulation which should follow effective strategies.

5.5.1 Marketing Strategies

The isolation of a certain strain of *B. bassiana* and confirmation of its effectiveness against some pest with relevant economic impact in the area represent the first step in the marketing process. This is typically carried out by researchers, who after applying for a patent can find a spin-off company to monetize their know-how. However, the most difficult part of the process is to make farmers understand how to use biocontrol products, compete with other companies, and fight against the already existing culture which certainly promotes chemical fertilizers and pesticides. A microorganism-based product for agriculture cannot be marketed as any other pesticide, and therefore, in order to increase sales successfully, it is critical to shift the mentality of farmers.

These are some suitable marketing strategies for this purpose:

(a) Free trials: This is a well known but effective strategy, which must be conducted by trained personnel and preferably in nonorganic crops. If the product works well for this kind of crops, organic farmers will immediately assume that the product will work also for their crops. However, when the tests are performed in organic crops, conventional farmers believe that the product will not necessarily work on their crop, because of the large amount of chemicals they apply.
(b) Creating a range of products (a system or a methodology): Farmers are much more likely to buy a full range of products or a system than an isolated product that is very different from the chemical products they are used to buy. In this way, they will understand that we have to change how we understand agriculture. It makes more sense for big and established chemical corporations to simply launch a new product (e.g., for the control of the tomato leaf miner), since they already have an existing range of products. Organic companies must create a new understanding of agriculture in order to be able to compete in the market and survive in a sector that is mainly controlled by few chemical corporations.

In the "product-by-product" fight, big corporations are unbeatable because of their huge marketing resources and distribution channels created for years. It is in the struggle between the old agriculture (chemistry) and the new agriculture (organic or integrated), where biocontrol companies are more likely to succeed.

(c) Starting with organic farmers and then expanding the business into nonorganic farmers: Obviously, organic farmers will be an easier target, but the organic farming market is not yet big enough to sustain the growth of new biocontrol companies. The real challenge for *B. bassiana*-based products is to compete with traditional pesticides. This is not a utopia, especially considering that these products are more sustainable and protect the immune system of the crops in the long term. The key for making this happen is the concept of integrated agriculture, which should convey the idea that it is not necessary for the farmer to choose between organic and nonorganic products, but they should rather integrate these two types of products in a single system. On the whole, this will be more sustainable and will ensure greater production in the long term.

5.5.2 Alternative Marketing Channels: *B. bassiana* in Earthworm Compost and Compost

B. bassiana is a fungus found in healthy soil, forming part of the immune system of the plant. Along with this fungus, many other microorganisms conform microbial communities that, together, create a biological balance capable of controlling many pests and diseases.

Many studies describe the presence of *B. bassiana* in vermicompost and compost (Anastasi et al. 2004). That is, there are other ways to ensure that *B. bassiana* is present in crops and thus benefit from their effects. Applying vermicompost in the planting substrate can achieve amazing results in controlling pests of great economic impact, such as the red spider mite (*Tetranychus urticae*) and root-knot nematodes (*Meloidogyne* spp.) (Arancon et al. 2002, 2007). This is particularly relevant from the marketing point of view. Given the strict regulations required to bring *B. bassiana* formulations to market, it is interesting for the business and consumer to know that the use of a natural and ecological fertilizer as vermicompost also ensures the presence of this fungus in the culture, which entails similar pest control benefits.

5.5.3 Marketing and Legal Framework

The legal framework for the marketing of *B. bassiana* formulations greatly varies depending on the country or region. However, in general, the greatest challenge is that there is no specific regulation for entomopathogenic biopesticides. On the contrary, these products are embedded in the existing regulations for plant protection products. This fact is criticized by many companies, since powerful and

toxic chemical pesticides are considered in the same category as organic and sustainable products.

Regarding the European Union, Regulation (EC) No. 1107/2009 of the European Parliament and the Council (October 21, 2009) establishes the basis for regulating the market of plant protection products. In short, this directive requires companies to conduct a series of experiments including field trials, trials with animals, plants, and insects. In practice, this process involves an average of 4–5-year evaluation period by the authorities, which does not guarantee approval. During this evaluation time, the sale of that product is not permitted. This is one of the major barriers for the marketing of *B. bassiana* in Europe and is not very different from the existing regulations in other regions of the world. This clearly benefits large corporations with big economic capacities and is detrimental for small producers of organic products.

5.6 Conclusion

The use of biopesticides represents part of the solution proposed by sustainable agriculture to the current chemical dependency. In this regard, *Beauveria bassiana* has proven its efficacy as biocontrol agent under different formulations. There is an increasing interest in developing safe and effective biopesticide products, which requires a multi-disciplinary holistic approach during the management of pest biocontrol solutions. On the other hand, specific regulations must evolve to evaluate systemic broader impacts of biopesticide products to assure their safety from both the human and ecosystem health point of view.

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Commercialization of Arbuscular Mycorrhizal Technology in Agriculture and Forestry

6

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Abstract

The ecto- and endomycorrhizal fungi are commonly occurring mycorrhizas and are very significant in relation to the growth of agricultural crops and forest trees. Mycorrhizal technology can advantageously be applied in agricultural and horticultural crops as well as forestry for better nutrient utilization offsetting ecological and environmental concerns by reduced chemical input use, disease management by reducing biotic stress by pathogenic fungi, and more effective land use management. However, even though the inoculation of plants with mycorrhiza is a familiar practice, the formulation of inocula with a dependable and steady effect under field situation is still a bottleneck for their wider use. The option of the technology for inocula production and of the carrier for the formulation is key to their booming application. In this review, we focus on the status of commercialization of mycorrhizal fungi as a gadget for enhancing plant growth and productivity.

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6.1 Introduction

Reduced cost and nondestructive means of achieving high productivity can be mutually reinforcing for the establishment of a viable low external input and sustainable farming system. This is basically achieved by an intervention which includes increase in the efficiency of crop production, diminution in agrochemical inputs, and an appraisal of the well-being and bioethical aspects in relation to societal perspective. However, to execute such a plan, we must develop plant systems with a vibrant rhizosphere that can competently forage and utilize soil nutrients present at critical levels (Brundrett 1991). Rhizosphere administration has emerged as an explanation in efforts to augment agricultural productivity and production since the finest use of nutrients, based on exploitation of microflora for indigenous and external application, can pick up crop productivity and curtail wastage of these nutrients, thus minimizing shock on environment leading to predisposition through the best possible production. The increasing demands for production of high-quality food using eco-friendly agricultural practices prompted the use of fertilizers based on useful microorganisms without deteriorating the natural resource base. In this context, biofertilizers would be a viable option for the farmers to increase the productivity per unit area. In this category mycorrhiza is getting prominence as simultaneous colonization by various mycorrhizal types and fungal species is common in 80–90 % of all known plant species covering bryophytes, pteridophytes, gymnosperms, and most of the angiosperms (Rakshit 2015; Rakshit et al. 2002). The fungus-plant interaction with a highly capable nutrient uptake systems and enhanced P storage abilities is capable to increase the nutrient absorbing exterior area away from the exhaustion zone of the root and can utilize organic nutrient resources. As a result of which, they are getting increasing attention for their role as biofertilizers, bioprotectors, and bioregulators (Pal et al. 2015, 2013, Parewa et al. 2014).

6.2 Mycorrhizal Synergy in Sustainable Agriculture and Forestry

Studies on the connections between plant, soil, and mycorrhizal fungi are shedding light on their interrelationships, thus providing new possibilities to utilize them for agriculture and forestry purposes. This symbiotic association results in distinct enhancement in crop growth and development mediated by enlarged effectual absorbing root surface (10X), nutrient absorption (N, P, Zn, Fe, B) and improved water transport ($\approx$2–3 10^{-5} mgs^{-1}) in plants resulting endurance to avert water stress, better outfitted to adverse effects of salt and also act to amplify intonation by

symbiotic N2 fixing bacteria and provide resistance against plant disease (Hodge 2000: Rai et al. 2013). Arbuscular mycorrhizal fungi can account for 5–50 % of the 50 % of the microbial biomass in the soil. Efficient mycorrhizal symbiosis can substitute more than 225 kg P_2O_5 ha^{-1} (Rakshit et al. 2002). In field studies, the growth and yield of crop species could be correlated to the mycorrhizal colonization rate. Mycorrhizal expertise can also be successfully introduced with the on hand forestry systems experienced to get better soil and crop productivity by allowing farmers to sustainably reduce the use of fertilizers and/or by enhancing plant endurance. Fitting mycorrhizal fungi can be included in nursery for raising mycorrhizal seedlings, and transfer of seedlings to the field is a simple inoculation technique currently appropriate in plantation crops and trees (Hilderbrandt et al. 2002). These practical applications of mycorrhizal fungi become visible to have beneficial effects on soil aggregation, thereby improving soil fertility, and may be important means of controlling wearing away of topsoil. Cultural practices that augment the activity of mycorrhizal fungi comprise of reduced tillage, crop rotations, cover crops, and phosphorus management tool. Awareness in the application of these products is going up due to the improvement in nutrient uptake efficiency and increasing societal anxiety for more green technologies in production and escalating expenses of agrochemicals (Bisen et al. 2015, 2016). Furthermore, mycorrhizal biofertilizers enjoy derived beneficial effects that would increase their convenience as bioinoculants.

6.3 Commercial Use of Mycorrhiza: An Overview on the Market and Products

Although mycorrhizal fungi are found in 85 % of all plant families and occur in many crop species for utmost growth, the extensive occurrence of these fungi in virtually all soils confines the pressing needs for inoculation with these natural linking fungi (Varma 1995, Newman 1988). Sophisticated scientific understanding on mycorrhizal symbioses recently enhanced commercial prospective for the implementation of mycorrhizal technology in agriculture plant production horticulture, forestry landscaping, phytoremediation in disturbed sites (Neill et al. 1991), and other facets of the plant market.

Production of mycorrhizal inocula is a multifarious procedure as it requires profit-making enterprises to develop the indispensable biotechnological proficiency and capacity to counter to permissible, ethical, educational, and saleable requirements. There are a broad variety of formulation types which include both liquid and solid and which can be tailored as per the requirement. Smart and high-class commercial mycorrhizal inoculum is now available from an array of sources. Consortium inocula containing mixtures of species of mycorrhizal fungi regularly give the best response (Barea and Jeffries 1995). Mycorrhizal inoculum comes in granular, powder, liquid, and tablet forms. Dry products comprise dusts, granules, and wettable powders. Dusts have particle size ranging from 5 to 20 mm and contain <10 % of an organism by weight. Granules are distinct masses 5–10 mm^3 in size, pellets are

>10 mm^3, and briquettes are large blocks (5 g) up to several cubic centimeters. These products include an inert carrier like charcoal, lignite, clay minerals (perlite, vermiculite, and bentonite), starch polymers, dry fertilizers, and ground plant residues. Above all, the most significant aspect is to get the mycorrhizal propagules in close proximity to the root systems of target plants. Presently a number of companies are producing mycorrhizal inocula for commercial purpose (Table 6.1). These may include varying amounts of different species of fungi, different percentages of

Table 6.1 Manufactures of mycorrhizal product across the globe

Country	Location	Company	Product
USA	Colorado	AgBio, Inc., Westminster	MycoApply
	Ohio	Accelerator Horticultural Products	MycorT AM
	California	Bio-Organics Supply, Camarillo	Mycorrhizal Landscape Inoculant (LA), Mycorrhizal Root Dip Inoculant (RD)
	Iowa	Becker Underwood, Ames	Myke®
	Arizona	BioScientific, Inc., Avondale	Ascend ST
	California	EcoLife Corporation, Moorpark	Bio Terra Plus
	MN	Sustane Corporate Headquarters	Sustane®
	Westminster, Colombia	AgBio, Inc.	AgBio-Endos™, AgBio-Ectos™
	Carpinteria, CA	Albright Seed Co./S & S Seeds	TurboStart™
	Ames, Iowa	Becker Underwood Biologicals	Rhizanova™
	Warrenton, Oregon	Bio-Oregon	BioVita™
	La Pine, Oregon	Bio-Organics	Bio-Organics™ Endomycorrhizal Inoculant (BEI), Bio-Organics™ Mycorrhizal Landscape Inoculant (LA), Bio-Organics™ Mycorrhizal Root Dip Inoculant (RD)
	Triadelphia, WV	First Fruits, LLC	EarthRoots™
	Olympia, WA	Fungi Perfecti	MycoGrow™, Plant Success™ Tabs
	Chino, CA	Gro-Power	GroLife™
	Parkland, FL	Hoodridge International	Mycoroot™

(continued)

Table 6.1 (continued)

Country	Location	Company	Product
	Sarasota, FL	Horticultural Alliance, Inc.	DIEHARD™
	Independence, MO	ROOTS, Inc.	MycorrhizaROOTS™, endoROOTS™, M-ROOTS™
	Ontario, Canada	Mikko-Tek Labs	
	Pittsburg, PA	Plant Health Care	Mycor™, Mycor™ Plant Saver™, Mycor™ Tree Saver™, Mycor™ Flower Saver, MycorTree™ Root Dip, MycorTree™ Ecto Spore Spray, PHC™ Colonize™ VAM Stimulant
	Red Hill, PA	Premier Enterprises Ltd.	MYKE™, MYKE™ Pro, MycorRise
	Lakeland, FL	Poulenger USA, Inc.	RUTOPIA + M™
	Salinas, CA	Reforestation Technologies International	Silva Dip™, AM120™, MycoPaks™
	Knoxville TN	The Tree Doctor	DieHard™ Injectable, DieHard™ Root Reviver
	West Lafayette, IN	Tree Pro	MycorTree™
Canada	Ontario	Mikro-Tek, Inc., Timmins	MIKRO-CONE®, MIKRO-VAM®
	Quebec	Premier Tech	Myke®
Chile	Sodegaura	Idemitsu Kosan Co.	R-10
France	Dijon	Biorize	Endorize
UK	Kent	Biological Crop Protection Ltd	Mycorrhizal inoculants
	Royston, Herts	MicroBio Ltd.	Vaminoc™
	Sittingbourne	PlantWorks Ltd.	Root Grow
	Lincoln	Crop Intellect Ltd.	Formulations of arbuscular mycorrhiza
	Mells	Zander Corporation	Arbuscular mycorrhiza
Spain	Zaragoza	Arvensis Agro	Arbuscular mycorrhiza
Belgium	Watou	Clemens Consult	Arbuscular mycorrhiza
Germany	Bitterfeld	Triton Umweltschutz GmbH	Myke Pro Endo AM
Poland	Końskowola	Mykoflor Włodzimierz Szałański	Mykoflor
Spain	Pedreguer	Odd Distributions	Root WebStar
Netherlands	Lelystad	Global Horticare	BioRoot
Czech Republic	Sázava, Lanškroun	Symbiom	Rhodovit, Symbivit®
Italy	Larino	Sacom	Seme

(continued)

Table 6.1 (continued)

Country	Location	Company	Product
India	Lansing, MI	VAMTech, Inc.	Mycoform® and Myconate®
	Chennai, TN	Biotrack Technology Pvt. Ltd.	RHIZAgold
	Andhra Pradesh	KCP Sugar and Industries Corporation Ltd.	Mycorrhiza VAM
	Ahmedabad	Cadila Pharmaceuticals, Ltd.	Josh
	Goa	Cosme Biotech	Shubhodaya
	Chennai, Tamil Nadu	ManiDharma Biotech Pvt. Ltd.	ManiDharma VAM
	New Delhi	TERI	Ecorhiza-VAM/ Nurserrhiza-VAM
	Bhopal, MP	Ambika Biotech	Root Care
	TN	Dr. Rajan Laboratories	Mycorrhizas
	Thanjavur, TN	TARI Biotech	Vesicular-arbuscular mycorrhiza
	Surat, Gujarat	Sundaram Overseas Operation	Vesicular-arbuscular mycorrhiza (VAM)
	Coimbatore, Tamil Nadu	T. Stanes and Company Limited	Solid formulations of arbuscular mycorrhiza
	Ahmedabad, Gujarat	Neesa Agritech Private Limited	Arbuscular mycorrhiza (powder and tablets)
	Una, HP	Majestic Agronomics Pvt. Ltd.	Arbuscular mycorrhiza
	Dwarka, Delhi	Krishidhan Seeds Pvt. Ltd.	Arbuscular mycorrhiza (powder)
	Coimbatore, TN	GreenMax AgroTech	Gmax VAM
Japan	Tokyo	Central Glass Co., Chemicals Section	Cerakinkong
	Tokyo	Idemitsu Kosan Co. Ltd.	R-10
Malaysia	Bangi, Selangor	Agri Hi-Tech Sdn	MycoGold, Myco-V
	Klang, Selangor	N-Viron Sdn Bhd	Mycorrhiza VAM
South Africa	Cape Town	Biocult	Biocult™

viable spores, as well as additives such as fertilizers and hydrogels. Some inoculants contain spores specific to particular species, while others contain a broad mixture. When choosing commercially produced inoculants, it is important to look out the specific plant requirement coupled with the existing soil conditions.

All through the last few years, there has been incredible explosion in market-producing mycorrhizal fungi inocula and allied services for the retail and wholesale segment. Clientele ranges from the familiar public and commercial growers to public and private institutions (Tiwari et al. 2002). The growth in the mycorrhizal industry is attributed to its growing body of scientific proof on the encouraging

effects of mycorrhizal fungi on plant health, fitness, and productivity coupled with mentoring by notable mycorrhizologist. In addition by tuning the appropriate inoculants, economic feasibility of mycorrhizal technology is gaining more prominence. Especially, there has been an appreciation in the market that mycorrhizal products offer a sustainable strategy to plant production in the present format of climate-resilient agricultural technology.

6.4 Commercialization of Mycorrhizal Technology

Mycorrhizal fungi have a colossal potential application in agriculture given a set of conditions that how the symbiosis helps plants to obtain nutrition. In spite of this, potential inoculation of crops is hardly ever seen, and mycorrhizas are only introduced consciously in a small number of industries (Adholeya 2003). Regardless of the possible yield increases, the moneymaking use of mycorrhizas has yet to take off. On the other hand, forestry is one of the industries that have completely approved the role of mycorrhizas in plant growth. The bulk of commercial timber comes from trees forming ectomycorrhizal links, and mycorrhizal symbioses are obligate in exotic trees. A number of other small industries habitually use mycorrhizal infection. The germination of orchid seedlings within the growth media will not take place without certain precise isolate of mycorrhizal inoculation resulting growers and small scale entrepreneurs of propagation unit for a rewarding impact. A latest expansion in the commercial use of mycorrhizas has been for land retrieval because of its capability to tolerate higher levels of heavy metals such as aluminum, iron, nickel, lead, zinc, and cadmium.

The fast-changing biotechnology environment, prejudiced by globalization, antagonism, financial pressures, and advancement of innovative technologies, has impacted the industry. In developed countries, it will come to the centre of interest as the demand for organic food continues to grow. The increases in growth and pathogenic resistance could be supplied by inoculating soil with mycorrhizas rather than using inorganic fertilizers, pesticides, and fungicides. Similarly, in developing countries, inoculating soil once with an appropriate fungal isolate could do away with the need for recurring applications of expensive fertilizers that farmers cannot willingly afford. Still the process of translating this idea into successful venture is getting obstacles due to lack of knowledge diffusion, vibrant consultancy services, and hope generators. As a result of which, it has landed up with just the linear reassignment of technology or intellectual property.

6.5 Constraints on the Commercial Use of Mycorrhizal Fungi

Even though during the last decade, mycorrhizal know-how reached a new height, the bottlenecks of these applications should be vigilantly measured. There is a definite drawback to the obligate character of the AM symbiosis resulting in its inability

to grow them in pure culture. According to current knowledge, they can only be grown with plants in restricted conditions, with the addition in the inoculants implying that they cannot readily be multiplied in laboratory media. At present, in bulk of the cases, mycorrhizal inoculum is produced as a non-sterile medium, either soil or some other non-sterile substrate, which contains spores, hyphae, and colonized root pieces. Because of this limitation, the present state of AM fungal inoculum production technology makes direct application of the fungus inoculum in wide areas of land burdensome and with poor cost-benefit ratio.

The rising sale of spurious bio-products and lack of vibrant quality control mechanism facilitate lower propagule number and their viability in many products. The issue of spurious mycorrhizal products also has adverse impact on the health of natural resource base. Further, the available commercial sources differ to a great extent in the nature of carrier medium, the number of species claimed to be present, and the number of active spores per unit weight or volume. The slower growth of fungi in comparison to other microorganisms makes its hurdle in popularization in large-scale agriculture.

6.6 Conclusion

Mycorrhizal fungi may be one option that can straightaway improve productivity in natural and managed ecosystems without deterioration of natural resource base and reduction of fertilizer costs and energy demands restoring economic efficiency and environmental security. If better strains of mycorrhizal fungi were developed, they could potentially advance growth of nearly all agronomic crops in a wide diversity of soils throughout the globe. Both endomycorrhizae and ectomycorrhizae are in commercial production on a small scale. Mycorrhizal inoculum production systems in a gel-based carrier have been proposed and manufactured by some elite scientists that are very concentrated and free from any microbial contamination. Such breakthroughs are necessary for mycorrhizal fungi to be practically used. The greatest obstacle in the commercialization of mycorrhizal fungi appears to be the lack of large-scale field tests under typical agroecology, adequate economics of the mycorrhizal fungi technology, and a growing unwillingness on the part of cultivators to switch from an energy-intensive system to a new, but cheaper, energy-conservative system using mycorrhizal fungi. Advocation and huge literacy drive of these geographically most widespread mutualistic associations could open up to the mycorrhizal industry an innovative approach for promoting a potential technology in sustainable agricultural system ensuring their effective quality product availability in the market. And with reference to commercialization strategy for mycorrhizal technology to be victorious, it must be competent, efficient, and spotlight on outcomes. Creating an entrepreneurial culture in the firm backed up by strong research infrastructure, network, and funding is another prerequisite. In years to come, it is highly likely that mycorrhizal biofertilizer can be a reliable partner with chemical inputs bringing benefits in agronomic, economic, and social perspectives.

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7 Microbial Consortial Products for Sustainable Agriculture: Commercialization and Regulatory Issues in India

Jegan Sekar, Rengalakshmi Raj, and V.R. Prabavathy

Abstract

Rhizosphere microorganisms directly and indirectly influence the composition and productivity of natural plant communities. Hence, belowground microbial species richness has been proposed as a predictor of aboveground plant diversity and productivity. Though research-based evidences clearly show the advantages of microbial consortia-based products due to their multifunctionality, limited attention is being given to develop quality standards for registration. This chapter focuses on the uses, commercialization, and regulatory issues of various bacterial consortia in sustainable agriculture.

Keywords

Consortia • Sustainable agriculture • Biofertilizers • Biopesticides • Rhizosphere

7.1 Introduction

Microbes are the most diverse communities on Earth that play a pivotal role in Earth's climatic, geological, geochemical, and biological process (Tringe et al. 2005; Xu 2006). The diverse genetic and functional groups of the soil microbial population exert a critical impact on soil function (Barea et al. 2005; Avis et al. 2008), particularly in the root–soil microhabitat referred to as rhizosphere which is considered as the hot spot for interaction between eukaryotes and prokaryotes (Jones and Hinsinger 2008; Hinsinger et al. 2009; Raaijmakers et al. 2009).

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Microbial interaction in the soil can be managed with low biotechnological inputs, to help sustainable and environment-friendly agro-technological practice (Azcón and Barea 2010; Ramos-Solano et al. 2010). The rhizosphere offers a complex microhabitat where root exudates provide a diverse mixture of organic compounds that are used as nutrients or signals by the soil microbial population (Brimecombe et al. 2007; Jones et al. 2009; Dennis et al. 2010; Bulgarelli et al. 2013) which results in a high degree of interaction between microbes, plant, and soil. Thus, understanding the function of microbial communities in the rhizosphere is of current research interest and has been extensively reviewed by many authors (Jones and Hinsinger 2008; Berg and Smalla 2009; Cavaglieri et al. 2009; Keswani et al. 2013; Unno and Shinano 2013; Vacheron et al. 2013; Chaparro et al. 2014; Gupta et al. 2015; Schlaeppi and Bulgarelli 2015; Bisen et al. 2015).

Rhizosphere microorganisms directly and indirectly influence the composition and productivity (i.e., biomass) of natural plant communities (Van der Heijden et al. 1998, 2008; Schnitzer et al. 2011). Hence, belowground microbial species richness has been proposed as a predictor of aboveground plant diversity and productivity (De Deyn et al. 2004; Hooper et al. 2005; van der Heijden et al. 2008; Lau and Lennon 2011). Wagg et al. (2011) further suggested that belowground diversity may act as an insurance for maintaining plant productivity under different environmental conditions.

Microbial groups residing in the rhizosphere include bacteria, fungi, archaea, algae, nematodes, protozoa, viruses, oomycetes, and microarthropods (Lynch 1990; Buée et al. 2009; Mendes et al. 2013). The bacterial groups like *Pseudomonas*, *Azospirillum*, *Methylobacterium*, *Enterobacter*, *Serratia*, *Arthrobacter*, *Azotobacter*, *Bacillus*, etc. lead the microbial population in the rhizosphere soil, followed by fungi, actinomycetes, and other groups (Gray and Smith 2005; Mendes et al. 2013; Nunes da Rocha et al. 2013). The overall interaction of the rhizomicrobiome and its function and impact on plant is represented in Fig. 7.1.

7.2 Plant–Microbe Interactions

Plant–microbe interactions in the rhizosphere depend on the function of the associated microorganisms based on which the microbes are classified as beneficial, deleterious, and neutral groups, and the bacteria that belong to the beneficial group are referred to as "plant growth-promoting rhizobacteria" (PGPR) (Kloepper et al. 1989). The PGPR are reported to enhance plant growth by a multitudinous mechanism which include production of plant growth-regulating substances (Kloepper 1993; Picard et al. 2000; Saravanakumar et al. 2008; Vyas and Gulati 2009; Farajzadeh et al. 2012; Santoyo et al. 2012; Bisen et al. 2016), phytohormones, suppression of plant pathogens through antibiosis (Couillerot et al. 2011; Sayyed and Patel 2011; Singh et al. 2011; Santoyo et al. 2012; Yin et al. 2013; Yokoyama et al. 2013; Sekar and Prabavathy 2014), nitrogen fixation (Franzini et al. 2010; Kathiravan et al. 2013; Mapelli et al. 2013; Sahoo et al. 2013), mineralization of organic phosphorus (Park et al. 2010; Sashidhar and Podile 2010), mediation of abiotic stress

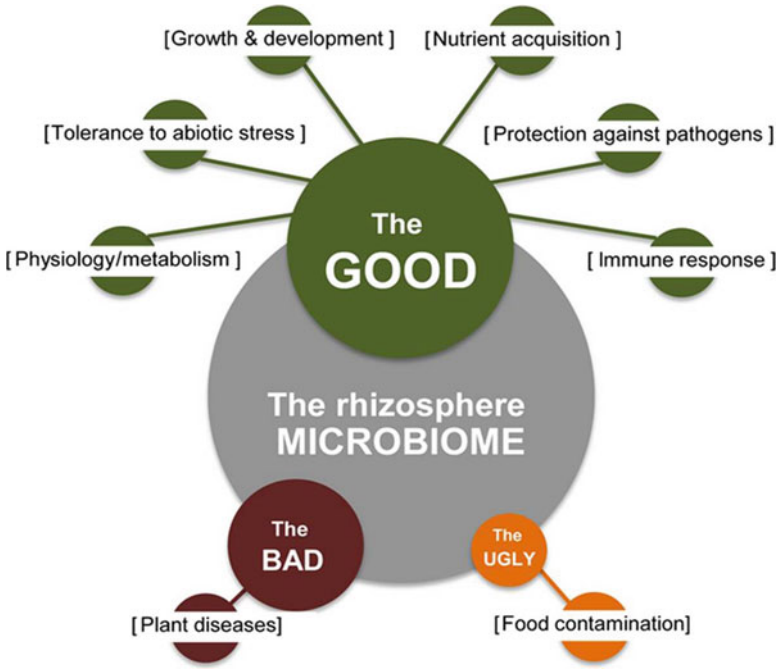

Fig. 7.1 Schematic representation of the functions and interactions of the rhizomicrobiome (Source: Mendes et al. 2013)

tolerance (Tringe et al. 2005; Zahir et al. 2009; Palaniyandi et al. 2013; Parihar et al. 2015; Shrivastava and Kumar 2015), production of phytoalexins/flavonoid-like compounds, and enhancement of mineral uptake (Parmar and Dadarwal 1999). The microbial community in the rhizosphere harbors members of few groups that adversely affect plant growth and health, viz., pathogenic fungi, oomycetes, bacteria, and nematodes (Raaijmakers et al. 2009; Damiani et al. 2012; Weller et al. 2012; Sekar and Prabavathy 2014).

Rhizosphere-associated copious beneficial microbial groups with multi-beneficial plant growth-promoting traits have been reported by many researchers (Raupach and Kloepper 1998; Picard and Bosco 2008; Ryan et al. 2008; Hartmann et al. 2009; Sekar and Prabavathy 2014; Viswanath et al. 2015; Krishnan et al. 2016; Raju et al. 2016). Bacterial groups secrete signaling molecules that influence bacterial gene expression and physiological behavior in a density-dependent manner termed quorum sensing (QS) (Zhang and Pierson 2001; Schuhegger et al. 2006; Liu et al. 2007; Viswanath et al. 2015); especially the rhizosphere regions were reported to harbor high N-acyl homoserine lactone (AHL) population (Elasri et al. 2001; DeAngelis et al. 2008; Viswanath et al. 2015). The QS-controlled phenotypes play a vital role for successful inter-/intra-gene and host interactions, whether symbiotic or pathogenic (Boyer and Wisniewski-Dyé 2009), and also influence interaction

with plants such as root colonization and induction of systemic resistance (Pang et al. 2008; Hartmann et al. 2014).

During the past few decades, the interaction between rhizobacteria and plants has been well explored and has resulted in the application of microbial products as crop inoculants (biofertilizers/biopesticides), for increased crop biomass and disease suppression. Combined application of potential PGPR strains is termed as microbial consortium (MC) which offers multi-beneficial plant growth-promoting traits and provides solution to underpinning problems like drought, salinity, increasing temperature, pest, and phytopathogenic infections in the agricultural system leading to global food safety and security. Microbial consortia are inoculants in a synergistic mixture which fulfill diverse functions in the rhizosphere and are the most promising contenders for solving challenges linked to sustainable eco-friendly agriculture (Jain et al. 2013).

7.3 Microbial Consortium as Biofertilizer and Biocontrol Agents

Currently agriculture is heavily dependent on mineral fertilizers and inorganic pesticides, and impacts of the continuous application are reflected in deteriorating soil health and increased resistance to pest and pathogens (Kumar et al. 2010; Cai et al. 2016). In the past 40 years, usage of nitrogen fertilizer has increased by sevenfold and pesticide usage by threefold. In the future these trends will continue unabated, as application of both inorganic fertilizer and pesticides is expected to increase by an additional threefold by 2050 which would cause unprecedented damage to the agroecosystem (Tilman et al. 2001).

Engineering the plant rhizomicrobiome is an alternative approach to increase soil health and enhance plant productivity (Jia et al. 2004; Wagg et al. 2011; Chaparro et al. 2012; Pindi and Satyanarayana 2012). Microbial interaction in the rhizosphere provides plants with multiple plant growth-benefiting traits and stress-tolerant traits apart from enhancing their own population and functions (Roesti et al. 2006; Jain et al. 2012; Wang et al. 2012; Jain et al. 2013; Singh et al. 2013; Thijs et al. 2014; Keswani et al. 2014; Armada et al. 2015). The inconsistency in performance of single microbial products in field application has emphasized the need for co-inoculation or consortia of microbial products (Bashan and de-Bashan 2005).

7.4 Bacteria–Bacteria Consortium for Plant Growth Promotion

Rhizobia and other PGPR share a common microhabitat, the root–soil interface, where interaction between different microbial groups was reported during root colonization. Co-inoculation of rhizobia with other PGPR enhanced nodulation and nitrogen fixation through the production of plant hormone, flavonoids, Nod factor, or enzymes in pigeon pea and other legumes (Tilak et al. 2006; Dardanelli et al.

2008; Remans et al. 2008; Medeot et al. 2010; Bansal and Srivastava 2012; Gupta et al. 2015). *Azospirillum*, a free-living diazotroph, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Serratia*, and *Enterobacter* are a few genera that have been successfully used with rhizobium as co-inoculants (Gaind et al. 2007; Remans et al. 2008; Cassán et al. 2009; Ahmad et al. 2011; Dashadi et al. 2011; Tajini et al. 2012; Ahemad and Kibret 2014; Gopalakrishnan et al. 2014). Besides the indigenous rhizobia community, inoculated diazotrophs like *Azospirillum* enhanced growth and yield in leguminous crops upon inoculation and increased fixed nitrogen quantity (Remans et al. 2008). Co-inoculation of *A. lipoferum* and *R. leguminosarum* bv. *trifolii* improved nodulation in white clovers, pigeon pea, and chickpea (Deanand et al. 2002). Most of the studies showed co-inoculation of *Azospirillum*, and *Rhizobium* significantly increased both the upper and total nodule number, acetylene reduction activities, faster 15 N dilution, and the total macro- and micronutrient mineral content as compared to other inoculants (Rodelas et al. 1996; German et al. 2000; Dardanelli et al. 2008; Askary et al. 2009; Cassán et al. 2009; Dashadi et al. 2011). Mehboob et al. (2013) extensively reviewed the effects of co-inoculation of rhizobia with various rhizospheric bacteria. *Azotobacter* was found to be a potential co-inoculant with rhizobium and enhanced the production of phytohormones and vitamins (Chandra and Pareek 2002; Qureshi et al. 2009; Dashadi et al. 2011; Akhtar et al. 2012). Co-inoculation of *G. intraradices*, *Pseudomonas striata*, and *Rhizobium* showed significant increase in plant growth, number of pods, and chlorophyll content in chickpea root rot (Akhtar and Siddiqui 2008).

Combination of *Rhizobium* with *Bacillus* strains was reported to improve root structure and nodule formation in bean, pigeon pea, and soybean (Halverson and Handelsman 1991; Srinivasan et al. 1997; Rajendran et al. 2008; Schwartz et al. 2013). Significant increase in root weight and seed yield of chickpea was reported upon inoculation of *Rhizobium* with *B. subtilis* OSU-142 and *B. megaterium* M-3 (Elkoca et al. 2010). Interaction of *Paenibacillus lentimorbus* NRRL B-30488 and *Piriformospora indica* DSM 11827 and their consortia with native rhizobia population in the rhizosphere of *Cicer arietinum* enhanced nodulation, thereby increasing plant growth (Nautiyal et al. 2010). When *R. tropici* CIAT899 was co-inoculated with *Chryseobacterium balustinum* Aur9, it resulted in increased root hair formation and infection sites leading to early nodule development and increased nodule formation (Estevez et al. 2009). A mixture of *Bacillus atrophaeus* and *Burkholderia cepacia* significantly reduced vascular wilt and corm rot in gladiolus diseases and enhanced plant growth by the elicitation of defense enzymes under field and greenhouse condition (Shanmugam et al. 2011).

Combined application of IAA-producing *Pseudomonas* sp. and *Mesorhizobium* sp. increased nodule formation and plant dry weight compared to *Mesorhizobium* alone inoculated and uninoculated (Malik and Sindhu 2011) plants. Similar effects were observed in chickpea upon co-inoculation with *Mesorhizobium* sp. and *P. aeruginosa* (Verma et al. 2013; Verma et al. 2014). Comparable plant growth-promoting effects along with antagonistic activities against *F. oxysporum* and *R. solani* were observed in chickpea by co-inoculation of *Mesorhizobium*, *Azotobacter chroococcum*, *P. aeruginosa*, and *T. harzianum*.

Consortia of *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3 showed improved yield of pigeon pea compared to treatment with individual isolates (Pandey and Maheshwari 2007). *Enterobacter* increased the nodule numbers in green gram when co-inoculated with *Bradyrhizobium* sp. (Gupta et al. 1998). Similar result was obtained when *Medicago truncatula* cv. *Caliph* was co-inoculated with *Pseudomonas fluorescens* WSM3457 and *Ensifer* (*Sinorhizobium*) *medicae* WSM419 (Fox et al. 2011).

Tomato plants inoculated with consortia of *Pseudomonas*, *Azotobacter*, and *Azospirillum* showed a maximum uptake of K by the shoots' (~7.97 %) enhanced fruit lycopene content and antioxidant properties (Ordookhani et al. 2010). Combined and individual application of *P. fluorescens* Pf1 and *B. subtilis* TRC 54 for the management of *Fusarium* wilt under greenhouse and field conditions improved defense-related enzymes peroxidase (PO) and polyphenol oxidase (PPO) and significantly reduced wilt incidence under greenhouse (64 %) and field (75 %) conditions (Akila et al. 2011). Application of the mixture of phloroglucinol-producing *P. fluorescens* F113 and a proteolytic rhizobacterium suppressed sugar beet damping-off (Dunne et al. 1998). Combination of different strains of *Pseudomonas* with iron-chelating and iron-inducing systemic resistance suppressed *Fusarium* wilt of radish compared to individual strain application (de Boer et al. 2003). Many strains of fluorescent pseudomonads and *Bacillus* sp. stimulated seed germination as well as root and shoot development in several crops (Rudresh et al. 2005). Root-nodulating *Sinorhizobium fredii* KCC5 and *P. fluorescens* LPK2e isolated from nodules of *Cajanus cajan* and disease-suppressive soil of tomato rhizosphere led to protocooperation as evidenced by synergism, aggressive colonization of the roots, and enhanced growth, suggesting potential biocontrol efficacy against *Fusarium* wilt in *C. cajan* (Kumar et al. 2010).

Co-inoculation of *B. subtilis* and *R. tropici* significantly reduced disease severity of bean root rot caused by *F. solani* f. sp. *phaseoli* and enhanced yield compared to control (de Jensen et al. 2002). *P. aeruginosa* PJHU15, *T. harzianum* TNHU27, and *B. subtilis* BHHU100 from rhizospheric soils triggered defense responses against *Sclerotinia* rot through elicitation of host defense response (Jain et al. 2012). Microbial consortium comprising of *P. fluorescens* (PHU094), *Trichoderma* (THU0816), and *Rhizobium* (RL091) activated physiological defense response in chickpea against collar rot pathogen *Sclerotium rolfsii* (Singh et al. 2013). Chickpea treated with consortium showed maximum activity of phenylalanine ammonia lyase and polyphenol oxidase and accumulation of total phenol content in chickpea than other treatments. Consortium of *B. subtilis*, *T. harzianum*, and *P. aeruginosa* showed improved yield along with disease reduction compared to either single or two microbe interaction upon challenge with the pathogen (Jain et al. 2015).

Interaction between *Streptomyces lydicus* WYEC 108 and *Rhizobium* was shown to promote growth in pea probably by nodule colonization of *Streptomyces* (Tokala et al. 2002). Nadeem et al. (2013) pointed out that the use of multi-strain microbial consortia is a better alternative for efficient performance, survival, and competence of the inoculum in natural environment and field conditions.

7.5 Arbuscular Mycorrhizal Fungi (AMF) and Bacterial Consortium for Plant Growth Promotion

Synergistic interaction between PGPR and AMF has been reported to increase yield and biomass in several plants under nursery and field conditions (Jia et al. 2004; Singh et al. 2008; Adesemoye et al. 2009; Singh et al. 2009; Wang et al. 2011; Tajini et al. 2012). Rhizosphere microorganisms either interfere or benefit mycorrhiza establishment (Pivato et al. 2009; Bonfante and Genre 2010; Miransari 2011; Tajini et al. 2012; Aroca et al. 2013). The beneficial effects exerted by the so-called mycorrhiza helper bacteria (MHB), a term referring to bacteria which enhance mycorrhiza formation, were reported by Frey-Klett et al. (2007). AMF and PGPR mycorrhiza helper bacteria interaction has beneficial implication in agriculture (Rabie et al. 2005; Aliasgharzad et al. 2006; Gamalero et al. 2008; Miransari 2011; Wang et al. 2011; Armada et al. 2015).

Co-inoculation of AMF with one or more PGPR has been reported to enhance growth and productivity in different crops (Dutta and Podile 2010; Reddy and Saravanan 2013). Several studies have reported the positive interactions between AMF and a wide range of PGPR, including phosphate-solubilizing bacteria, nodule-forming N_2-fixing rhizobia, and free-living *Azospirillum* spp., *Bacillus* sp., and *Pseudomonas* sp. (Gamalero et al. 2008; Singh et al. 2009). Co-inoculation of AMF and PGPR was reported to have a synergistic effect on plant growth especially under growth-limited conditions (Vivas et al. 2003a, b). Among the microbial groups, PGPR and AMF promote activities which improve agricultural development (Barea et al. 2005). The bioinoculants AMF and PGPR had a significant effect on grain quality, for instance, the phosphorus content doubled in the bioinoculant-applied rainfed wheat, both in greenhouse and field experiments (Roesti et al. 2006). Co-inoculation of AM fungi and biocontrol agents resulted in the suppression of soilborne pathogens such as *Fusarium* and *Rhizoctonia*. Enhanced bioprotection results by the combination of mechanism exhibited by individual organisms, such as competition, altered root exudates, morphological changes in the root system, antibiosis, and activation of plant defense response (Saldajeno et al. 2008).

The AM symbiosis in legumes and its role in improving nodulation and nitrogen fixation by legume–rhizobia association either at the colonization or symbiotic functional stage have been reported (Lesueur et al. 2001; Lesueur and Sarr 2008; Azcón and Barea 2010). Positive effects of the combination of mycorrhizal fungi and/or PGPR on plant growth and plant health as biostimulators, biofertilizers, and bioprotectants have been described by many authors (Barea et al. 2002; Azcón and Barea 2010; Sharma et al. 2016). Arbuscular mycorrhizal fungi (AMF) and rhizobia are the most important symbionts for the plant to acquire nutrients efficiently and to promote growth. Tajini et al. (2012) used *Glomus intraradices*, a potential P mobilizer, and *R. tropici* CIAT899, a nitrogen fixer, to increase the phosphorus-use efficiency for symbiotic nitrogen fixation in common bean (*Phaseolus vulgaris* L.). Co-inoculation of rhizobia and arbuscular mycorrhizal fungi (AMF) promoted growth of soybean under low phosphorus and nitrogen conditions, indicated by increased shoot dry weight (Wang et al. 2011).

Boby and Bagyaraj (2003) reported the effect of *G. mosseae*, *P. fluorescens*, and *T. viride* consortium against soilborne root-rot wilt caused by *Fusarium chlamydosporum* in *Coleus forskohlii*. Consortia of *T. viride* and *G. mosseae* decreased the disease severity and enhanced maximum growth compared to other combinations. Another study by Singh et al. (2009) reported the most effective suppression of root-rot wilt in *C. forskohlii* by a consortium of AM fungus *G. fasciculatum* and *P. fluorescens*. Though in both the reports consortium showed enhanced biocontrol activity against root-rot wilt, the combination of efficient compatible strains in the consortium contributes to more efficient control of the pathogen.

Consortium of *Bradyrhizobium* sp. BXYD3 and *G. mosseae* significantly decreased the severity of *Cylindrocladium parasiticum* incidence in soybean by altering the pathogen defense-related (PR) genes *PR2*, *PR3*, *PR4*, and *PR10* expression level (Gao et al. 2012). A combined bio-inoculation of 2,4-diacetylphlorogluci nol-producing PGPR strains and AMF synergistically improved the nutritional quality of the grain in three Indian rainfed wheat without negatively affecting mycorrhizal growth (Roesti et al. 2006), and in addition it stimulated both mycelial development and spore germination in *G. mosseae* and enhanced root colonization in tomato (Barea et al. 1998). Combined application of AM fungus *F. mosseae* with *Paenibacillus* and *Pantoea* spp. enhanced all the biometric parameters in French bean especially the total shoot dry biomass and fruit yield.

Rhizobium and AMF co-inoculation increased leaf area and biomass production in broad bean (*Vicia faba*), AMF colonization increased the supply of P, and *Rhizobium* facilitated N accumulation (Jia et al. 2004). The application of a consortium of microbial inoculants such as mycorrhiza and *Azospirillum brasilense* effectively increased plant growth and enhanced the ability of plants to alleviate drought and nutrient stress (Azcón and Barea 2010). AM fungus *G. intraradices* enhanced growth, photosynthetic efficiency, and antioxidative response in rice against drought stress (Ruiz-Sanchez et al. 2010).

Kamal et al. (2016) evaluated the impact of *Streptomyces labedae* (SB-9), *Streptomyces flavofuscus* (SA-11), *Pseudomonas poae* (KA-5), *P. fluorescens* (KB-7), and *G. intraradices* consortium combination which showed pronounced increase in the finger millet plant growth under drought condition.

Seed priming with consortia of *T. harzianum* and fluorescent pseudomonas decreased the *Fusarium* wilt incidence, increased seed germination by 22–48 %, and reduced the germination period (Srivastava et al. 2010). The enhanced performance of microbial consortia compared to single inoculation is reported in several crops including legumes (Antoun et al. 1998; Valdenegro et al. 2001; Ane et al. 2004; Bagyaraj and Kehri 2012; Bagyaraj 2014). Consortium product "Shu Dekang" showed significant control of several phytopathogenic infestations like leaf speck disease, banana wilt, and root-knot disease (Zheng et al. 2010). Thus, PGPR consortia with multiple functions provide multiple growth-promoting and stress-tolerant benefits in plants.

7.6 Microbial Consortium for Abiotic Stress Alleviation

The global climate is a great challenge for the agricultural sector, as predicted increases in salinity, drought, and rising temperature cause abiotic stress in the plant which reduce crop productivity (Grover et al. 2011; Larson 2013). About 60 % of the global geographical area faces soil degradation either by waterlogging or salinity or alkalinity, which threatens food security, the situation being worse in higher rainfall areas where waterlogging follows shortly after the rains (Singh 2000).

Plant-associated microbial communities have received considerable attention for their ability to confer many of the same benefits to crop productivity and stress resistance as have been achieved through plant breeding programs (Mayak et al. 2004; Barrow et al. 2008; Marulanda et al. 2009; Mapelli et al. 2013). Microbial symbionts are capable of conferring multiple stress tolerance against both abiotic and biotic stress (Mayak et al. 2004; Rodriguez et al. 2008) benefits in both monocot and dicot crop species (Timmusk and Wagner 1999; Redman et al. 2002; Zhang et al. 2008).

Application of microbial inoculants specially consortia will be one of the solutions to alleviate plant abiotic stress and enhance plant growth and productivity under stress conditions (Yang et al. 2009; Jain et al. 2013). Multiple beneficial PGP and abiotic stress-resistant strains, efficient 2,4-DNT-degrading consortia composed of *Burkholderia*, *Variovorax*, *Bacillus*, *Pseudomonas*, and *Ralstonia* spp., have been reported (Shirley et al. 2000; Snellinx et al. 2003) to enhance the root length of *Arabidopsis* under 2,4-DNT stress, by doubling the root length within 9 days (Thijs et al. 2014).

Co-inoculation of *A. brasilense* with *R. tropici* on bean relieved negative effects of salt stress and nod gene transcription (Dardanelli et al. 2008). Microbial consortium comprising of *P. fluorescens* (PHU094), *Trichoderma* (THU0816), and *Rhizobium* (RL091) enhanced the expressions of defense systems like antioxidant enzymes superoxide dismutase and peroxidase activities (Singh et al. 2013) under stress. The response of rice plants to inoculation with an AMF and *A. brasilense* consortia under drought stress conditions was due to enhanced ascorbate accumulation. The effect of *A. brasilense* was pronounced only when mycorrhizal colonization was established; thus, the bacterial and fungal consortia were responsible for the protection of plant against plant pathogens (Ruiz-Sanchez et al. 2011). PGPR consortium of endophytic bacterium *P. pseudoalcaligenes* in combination with *B. pumilus*-treated plants showed increased concentrations of NPK and reduced concentrations of Na and Ca in paddy under saline conditions (Jha and Subramanian 2013). Co-inoculation of *P. fluorescens* Aur6 and *Chryseobacterium balustinum* Aur9 in three field experiments induced systemic resistance in rice against rice blast and increased rice productivity and grain quality under saline conditions (Lucas et al. 2009).

Under drought stress cucumber seedlings treated with consortium product of Shu Dekang containing *B. cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21 showed enhanced photosynthetic efficiency, less wilt symptoms, decreased leaf monodehydroascorbate (MDA), increased leaf proline content, enhanced induced

systemic tolerance, and superoxide dismutase activity. Downregulation of the expression of the genes *cAPX*, *rbcL*, and *rbcS* encoding cytosolic ascorbate peroxidase and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large and small subunits was observed (Wang et al. 2012).

Consortium of *P. polymyxa* and *R. tropici* increased plant growth, nitrogen content, and nodulation of common bean (Figueiredo et al. 2008). The co-inoculation of *G. intraradices* and *R. leguminosarum* protected bean plants under drought conditions in semiarid region by increase in plant biomass, grain yield, and several antioxidant enzyme activities in the host plants (Zahran 1999; Valdenegro et al. 2001; Aroca et al. 2007).

The consortium of *Pseudomonas mendocina* and *G. intraradices* protected and enhanced plant growth in *Lactuca sativa* L. cv. by the production of antioxidant enzymes such as superoxide dismutase, catalase and total peroxidase, phosphatase, and nitrate reductase in leaves (Kohler et al. 2008). Under salinity stress inoculated plants showed significantly higher shoot biomass and glomalin-related soil protein (GRSP) compared to uninoculated plants (Kohler and Caravaca 2010). *A. brasilense–Rhizobium* combination enhanced the growth of *P. vulgaris* under salt stress by increasing nodulation, flavonoid, and lipochitooligosaccharide production (Dardanelli et al. 2008; Smith et al. 2015). Gamalero et al. (2008) showed the impact of ACC deaminase in cucumber treated with PGPR *P. putida* UW4 and *Gigaspora rosea*, where synergistic action was reflected on plant biomass, root length, total leaf area, and increased photosynthetic performance index. *Zea mays* co-inoculated with *Rhizobium* and *Pseudomonas* under salinity conditions showed increased production of proline and maintenance of relative water content of leaves, reduction in electrolyte leakage, and selective uptake of K ions (Bano and Fatima 2009).

Consortium of *B. thuringiensis* and AMF reduced the oxidative damage to lipids and increased drought-induced proline in *Zea mays* under stress. *B. thuringiensis* increased plant nutrition, and AMF enhanced the stress tolerance/homeostatic mechanisms, by regulation of plant aquaporins with many putative physiological functions (Armada et al. 2015). *B. subtilis* and *Arthrobacter* sp. co-inoculation alleviated adverse effects of 8 % soil salinity on wheat and enhanced the dry biomass, total soluble sugars, proline content, and antioxidant enzymes in wheat leaves which decreased under salinity stress (Upadhyay et al. 2012). Prasanna et al. (2015) used cyanobacterial inoculants *Anabaena–Azotobacter* biofilm and *Anabaena* sp.–*Providencia* sp. to enhance the Zn mobilization in maize hybrids and elicit plant defense response. Both consortia were found to enhance the activity of defense enzymes such as polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia lyase (PAL) in roots, with a positive correlation of Zn concentration in the flag leaf.

7.7 Commercialization and Registration of Biofertilizers in the World

Unlike in microbial biopesticide category, microbial consortia are acknowledged and promoted in the case of biofertilizers in many countries. In the USA and EU, currently there is no specific legal definition for biofertilizers. In EU, all microorganisms irrespective of its principle action are included as possible products for

organic production as per the European Commission Regulation No. 889/2008 (Malusa and Vassilev 2014). But, India has a comprehensive legal framework on biofertilizers. The Ministry of Agriculture issued an order in 2006 (subsequently amended in 2009) categorizing the biofertilizers under Essential Commodities Act of 1966 and brought under Fertilizer Control Act 1985. Under this act production and marketing standards were specified for different biofertilizers. As per the definition of biofertilizer under the Indian act, it does not specify any microbial consortia, while the proposed concept of microbial consortium under the legal provision regulating the production and marketing of biofertilizer in EU was specified in the definition itself (Malusa and Vassilev 2014).

7.8 Biofertilizer Commercialization and Regulatory Issues in India

Biofertilizer commercialization began with the rhizobia product in the year 1895 by Nobbe and Hiltner under the brand of "Nitragin." In India, N. V. Joshi first started the commercialization of rhizobium for the growth promotion of leguminous plant (Rivas et al. 2015). During its ninth five-year plan, the Ministry of Agriculture initiated the popularization and promotion of biofertilizer production, developing standards for different biofertilizers, training, and utilization by launching National Project on Development and Use of Biofertilizers (NPDB), and a National Biofertilizer Development Centre was established, with six regional centers (Ghosh 2004). The government of India and state governments took several measures for promoting the production of biofertilizers by providing grants and subsidies at different levels.

The Ministry of Agriculture passed a new decree on the control of biofertilizer production and marketing standards with regard to different kinds of microorganisms. The product should fulfill seven quality parameters like physical form, minimum count of viable cells, contamination level, pH, particle size in the case of carrier-based materials, maximum moisture percent by weight of carrier-based products, and efficiency character. In bacterial bioproducts the minimum viable cells to be maintained is 5×10^7 CFU g^{-1} for solid carrier or 1×10^8 CFU ml^{-1} for liquid carrier. For products containing mycorrhizal fungi, at least 100 viable propagules must be present per gram of product. Nitrogen-fixing efficiency of biofertilizer product should be capable of fixing at least 10 mg N g^{-1} of sucrose consumed and for phosphate solubilization product a zone of solubilization at least 5 mm in a media. AMF products should provide 80 infection points in roots g^{-1} of inoculum (Ministry of Agriculture 2009).

Markets and Markets (2015) report shows that the biofertilizer market is projected to grow at a CAGR of 14.0 % from 2015 to 2020 and is expected to reach US $1.88 billion by 2020. Leading players in the biofertilizer market include Gujarat State Fertilizers & Chemicals Ltd. (India), Novozymes A/S (Denmark), Rizobacter Argentina S.A. (Argentina), Camson Bio Technologies Limited (India), and Lallemand, Inc. (Canada) (RNR Market Research 2014). Biofertilizer market in

Asia is strongly influenced by the government and its policies to promote sustainable and green agriculture. Around US $1.5 billion has been spent on the development of biofertilizer and biopesticide products (Rivas et al. 2015). Currently there is an increase in organic agriculture practice in the country with around 1,000,000 ha under organic cultivation (Keshri 2016).

In India, around 100 public and private companies are involved in biofertilizer production, and the list of a few companies and their consortial products are listed in Table 7.1 (Rivas et al. 2015). Biofertilizer production and consumption have gained importance in the recent times in India (Pindi and Satyanarayana 2012). The average consumption in the country is about 45,000 t per annum, while its production is less than half of the consumption. The maximum production capacity lies in Agro Industries Corporation followed by state agriculture departments, National Biofertilizers Development Centres, State Agricultural Universities, and private sectors (Mazid and Khan 2014).

7.9 Commercialization and Registration of Biopesticides in the World, Asia, and India

Worldwide the use and demand for biopesticides are rising due to the increased awareness of pesticide residue-free crops. The global-level estimate for microbial products in 2014 was US $ 2,183 million which is projected to double by US $ 4556 million in 2019 with a CAGR of 15.3 %. Of the several microbial types, the bacterial segment accounted for the largest share (US $1.6 billion). Similar to biopesticides, market for biofertilizers at global level is projected to reach US $1.88 billion by 2020 at a CAGR of 14.0 % from 2015 to 2020 (Markets and Markets 2015).

Globally, more than 200 biopesticide active ingredients are registered, and 700 products are available in the market. In the case of India, 15 biopesticides were registered as on 2008 under IA (1968), and its market share is only 4.2 % of the overall pesticide market; however, it is predicted to increase at an annual growth rate of 10 % (Suresh 2012). While its growth was multifold during the past years, NAAS (2013) reported around 400 registered biopesticide active ingredients and over 1250 actively registered biopesticide products in Indian markets. It shows the awareness among farmers as well as policy support of the government to use the ecologically safe products for pest management. However, there is no specific mention about microbial consortium among 400 registered biopesticides individually.

At the international level, the regulatory frameworks differ widely among different countries. In the USA, biopesticide production is institutionalized under a separate division as “Biopesticides and Pollution” within the Environmental Protection Agency (EPA). To maintain the quality, it specified good laboratory practices regulatory testing for microbial biopesticides in 1983 as EPA M guidelines. Following the line, in 1996 the Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF) harmonized its system with guidelines of EPA. Similarly in Europe, biopesticides are evaluated through the European Pesticide Regulation EC No. 1107/2009 which promotes the production of less harmful substances, and it has

Table 7.1 List of few representative commercial consortial products

S. no	Product	Consortia	Company	Country
1.	Life®	PGPR consortia	Biomax	India
2.	Biomix®	PGPR consortia	Biomax	India
3.	Biozink®	PGPR consortia	Biomax	India
4.	Biodine®	PGPR consortia	Biomax	India
5.	Jet 9	PGPR consortia	Sivashakthi Bio Planttec Ltd.	India
6.	Calosphere	PGPR consortia	Camson Bio Technologies Ltd.	India
7.	Calspiral	*Azospirillum* + PGPR	Camson Bio Technologies Ltd.	India
8.	Symbion-N	*Azospirillum* + *Rhizobium* + *Acetobacter* + *Azotobacter*	T. Stanes & Company Ltd.	India
9.	Bio Power	*Azospirillum* + *Azotobacter* + PSB + VAM	SKS Bioproducts Pvt Ltd.	India
10.	Bio Super	*Pseudomonas* + *Cellulomonas* + *Bacillus* + *Rhodococcus*	SKS Bioproducts Pvt Ltd.	India
11.	Premium EMC	PGPR consortia	International Panaacea Ltd.	India
12.	Nodulator® N/T	*B. subtilis* MBI 600 + *B. japonicum*	BASF Canada, Inc.	Canada
13.	Nodulator® PRO	*B. subtilis* + *B. japonicum*	BASF Canada, Inc.	Canada
14.	BioBoots®	*Delftia acidovorans* + *Bradyrhizobium* sp.	Brett-Young Seeds	Canada
15.	EVL Coating®	PGPR consortia	EVL, Inc.	Canada
16.	BioAtivo®	PGPR consortia	Instituto de Fosfato Biológico (IFB) Ltda.	Brazil
17.	BioJet®	*Pseudomonas* sp. + *Azospirillum* sp.	Eco Soil Systems, Inc., San Diego, CA	USA
18.	BioYield	*B. subtilis* + *B. amyloliquefaciens*	Gustafson, Inc., Dallas	USA
19.	TagTeam®	Rhizobia + *Penicillium bilaii*	Novozymes	USA
20.	VitaSoil®	PGPR consortia	Symborg	Spain

been promoting the registration of low-risk products for pest control through (2009/128/EC) simple and transparent registration protocols (Villaverde et al. 2014). Canada follows only the safety test and the rest of the countries need data of both safety and efficacy tests. The EPA, JMAFF, and EC regulations toward biopesticides are developed in such a way that it requires less data when compared to chemical products and reduced the time to process the registration applications. In this context, the International Organization for Biological Control of Noxious Animals and Plants (2010) carried out a global-level review on the use of biopesticides and regulatory measures. It stressed the need for streamlining the registration process through harmonizing data requirements and protocols for risk assessments. In India, any microorganisms used for pest and disease management require registration for both production and sale with the Central Insecticides Board (CIB) of the Ministry of Agriculture as per the Insecticides Act (IA), 1968, of the Government of India (GOI) and Insecticides Rules, 1971, which were recently replaced by the Pesticides Management Bill 2008. The biopesticides are considered as generally regarded as safe (GRAS) under this act, and to promote its production and use, it provides the benefit of priority in processing of registration as well as provisional registration. Thus, the producers can register the product either for regular registration under section 9 (3) or for provisional registration under section 9 (3B) of the IA. While applying for registration, the data on product characterization, safety, toxicology, efficacy, and labeling are necessary. In addition to the priority and provisional registration for biopesticides in the Act, the registration protocols are made easier and accept generic data for any new products containing strains which are already registered. Such affirmative clauses are inbuilt in the Act which shows the interest of the government in promoting the safe products for pest management similar to other countries. In order to regulate the commercial production of these products, the Government of India established four different bodies to regulate the biopesticide production. The Central Insecticides Board (CIB) is involved in developing appropriate policies, and the Registration Committee (RC) is responsible to register the products for production. Whereas the Central Insecticides Laboratory (CIL) is in charge to monitor the quality of the products available in the market, finally the State Department for Agriculture (SDA) issues the manufacturing license and performs quality check. However, coordination among the four bodies plays a vital part in ensuring the registration and availability of quality products in the market. Recently, efforts were taken to harmonize the IA of 1968 with the Organization for Economic Cooperation and Development (OECD) during 2000s on the methods and approaches to assess biological pesticides. On this basis, CIB has rationalized the guidelines and data requirements for registration and infrastructure necessary for production of the biopesticides (NAAS 2013). However, research studies on how the harmonization eased the process of registration are yet unavailable. On the other side, as per the notification dated March 26, 1999, of the Central Insecticides Board, Ministry of Agriculture, biopesticide was put under the Insecticide Schedule Act 1968, and hence, the generation of toxicological data became a prerequisite for the registration of biopesticide. In spite of the relatively abundant number of patents for microbial pesticides, the number of commercial applications has not been as

dramatic as expected due to the high cost involved in toxicologic analysis, biosafety, and environmental concerns (Montesinos 2003).

7.10 Registration and Regulations for Microbial Consortia

Though research-based evidences clearly show the advantages of microbial consortia-based products due to their multifunctionality, limited attention is being given to develop quality standards for registration (Jain et al. 2013). NAAS (2013) reiterated that microbial consortium-based products require meticulous calibration in terms of cultural methods and their microbial composition in the product cycle. It is well understood that the evaluation of the efficacy of biofertilizer-based microbial consortia is complex due to its multiple mechanisms of action, viz., plant growth protection, stimulation, etc. However, farmers and market agencies prefer microbial consortia-based products due to its practical easiness in use, economic reasons, and multifunctional properties. Hence, initiatives have been taken to address the concern at different levels. The overall matter appears even more complex as some microorganisms either as single or as member of the microbial consortia can have both effects as biofertilizers/bioeffectors and plant protectants. The study of Malusa and Vassilev (2014) suggested that the principal function of the product can be taken for classification and labeling considering its potential environmental risks and study of its ecotoxicology and impact on environment when other products such as additives or nanomaterials are included in the formulations.

7.11 Conclusion

Though the performance of the PGPR and its consortia has been proved to promote plant growth and enhance productivity in the field conditions by several strains in different crops, the use of these products has not been popular among farmers due to several reasons such as (1) lack of awareness among farmers and (2) availability and supply of quality bioproducts. A survey conducted by Srinivas and Bhalekar (2013) reported the communication gap that exists between farmers and manufactures, miscommunication about the quality of the product, and sustainability of biofertilizer as the major hurdle. In natural conditions and in disease-suppressive soil, the existence of mixture of microbial antagonists (Lemanceau and Alabouvette 1991) has been reported. Hence, augmentation of compatible strains of PGPR to infection court will mimic the natural environment and could broaden the spectrum of biocontrol against different plant pathogens. Efficiency of biocontrol agents could be increased by the development of compatible strain mixtures of different biocontrol organisms by considering the following norms (Raupach and Kloepper 1998). While developing a consortial formulation, the following needs to be addressed: (1) compatible strain combination that differs in the pattern of plant colonization, (2) compatible strain combination with broad spectrum of action against different plant pathogens, (3) compatible strain combination with different modes

of action, and (4) compatible strain combination of genetically diverse group to adapt to different pH, moisture, temperature, and relative humidity. The use of microbial inoculants must take into account the importance of retaining microbial diversity in the rhizosphere and in achieving realistic and effective biotechnological applications. Molecular biology-based approaches by developing molecular markers to analyze the impact of the introduced isolate on the microbial diversity and community structure and to predict responses to microbial inoculation/processes in the environment (ecological engineering) are essential. Further studies must address the consequences of the cooperation between microbes in the rhizosphere under field conditions to assess their ecological impacts and biotechnological applications. In this context further research and efforts are needed to promote the use of microbial consortia considering its multifunctional characteristics; at the same time, quality standards for the crop-specific/soil property-specific potential combination of microbes have to be generated to ease the registration process. While developing such standards, harmonization at global level would help to speed up the process and reduce the time and resources which are vital to promote quality products.

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8 Agriculturally Important Microorganisms as Biofertilizers: Commercialization and Regulatory Requirements in Asia

Vachspati Pandey and K. Chandra

Abstract

Biofertilizer refers to the different formulations of living preparations of agriculturally important beneficial microorganisms (bacteria/fungi) with certain desirable physiological and behavioral characters which are utilized for crop nutrition management programs. The major attributes of quality of different biofertilizers is well established and efforts are being made to regularize the production and marketing of these biofertilizer products. During the last two decades, biofertilizer production and marketing exhibited phenomenal growth in most of the Asian countries. Now time has come to form some set of rules assuring the quality of biofertilizers available in the market. In any microbial biofertilizer formulation, viable cell count is the crucial parameter, while the ability of organisms to fix nitrogen or solubilize phosphorus, potassium, zinc, etc. is important efficiency characters being considered in formulating the quality standards for biofertilizers. In the present chapter, the situation of biofertilizer quality control and regulatory mechanism in Asia are discussed.

Keywords

Biofertilizers • Quality regulations • Production standards • Agriculturally important microorganisms

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8.1 Introduction

Agriculturally important microorganisms as biofertilizers are being used since long and have a vital role in integrated nutrient management (INM) system. They not only offer the required nutrients to plants but also help in maintaining soil health (Pandey et al. 2001). Biofertilizers are cost-effective, eco-friendly natural inputs providing alternative source of plant nutrients, thus increasing farm income by providing extra yield and reducing input cost (Bhattacharyya et al. 2002; Ram et al. 2016). During the last decade, organic farming movement helped the biofertilizer technology to gear up globally as an important tool in organic nutrient management.

Biofertilizers are unique, cost-effective, and eco-friendly alternative to synthetic fertilizers which improve not only the crop productivity but also improve soil health in sustainable manner (Bisen et al. 2015). Biofertilizers have become a global reality with more and more acceptance from mid- and small-scale farmers especially from Asia and South America (Keswani et al. 2013).

8.2 Status and Potential of Biofertilizers in Asia

While Asia is one of the biggest producers and consumers of biofertilizers, there are still few untied threads which pose a major biosafety concern for environmentalist. Firstly there is a used difference in the guidelines for mass production and commercialization in the developed, developing, and underdeveloped countries. These unparallel regulatory measures pose a huge setback for intercontinental trade. Moreover, the expanding demand for organically grown food supplied provides an excellent opportunity for developing agro-based economies specifically in Asia. The growing demand increases the responsibility of the governmental regulatory bodies, industries, and academia to employ strict regulatory guidelines for production of high-quality biofertilizers, which have the desired effects as advertised.

Easy regulatory and registration framework based on advanced R&D in countries like China, India, Korea, Japan, and Taiwan has tremendously helped the acceptance of biofertilizers. However, successful adoption of biofertilizers largely depends upon the quality of the biofertilizer product and also sensitization and training to the farmers (Banayo et al. 2012).

In Japan, the Tokachi Federation of Agricultural Cooperative (TFAC) in Hokkaido produces rhizobium biofertilizers under the trade names Mamezo for soybean and Azuki for bean (Sheng 2005; Sheng and He 2006). Central and local government agencies in Taiwan support popularization of biofertilizers including rhizobium, P-solubilizing bacteria, and mycorrhizal inoculants for horticultural crops. From 1987 to 2006, enough inoculants were produced to inoculate approximately 65,091 ha of farmland. Farmer's annual income also increased significantly from using biofertilizers (Liu et al. 2012; Njira 2013).

In Vietnam, farmers in the Mekong River Delta have been using BioGro in rice, and this resulted in disease reduction and higher yields (Jones and Darrah 1994).

In U Thong District of Suphan Buri Province in Bangkok, Thailand, farmers demonstrated the application of indigenous knowledge in the development of biofertilizers. In China, most of the farming community regularly integrates biofertilizers in their farming regime. The quality of biofertilizers in China is based on eight essential parameters, viz., number of viable cells, pH, contamination, granule size, carbon and moisture content, appearance, and date of expiry (Suh et al. 2006). In Pakistan the major constraints in the widespread use of biofertilizers are irregular field performance, lack of regulatory framework, and lack of publicity (Naveed et al. 2015).

Quality enforcement measures, which were absent till 2006, have been addressed to some extent by incorporation of some formulations under the provisions of Fertilizer (Control) Order 1985 (Yadav and Chandra 2014). For each group of microorganisms, a detailed procedure for the quality control including registration, scale of sampling, and methods of analysis is also specified. Seven groups of microorganisms are considered including *Rhizobium*, *Azotobacter*, *Azospirillum*, phosphate solubilizing biofertilizer, potassium solubilizing biofertilizer, zinc solubilizing biofertilizer, and mycorrhizal (AM) biofertilizer.

8.3 Legal Definitions of Biofertilizers

A biofertilizer can be defined as the formulated product containing one or more microorganisms which enhance the nutrient status (and the growth and yield) of the plants by either replacing soil nutrients and/or by making nutrients available to plants and/or by increasing plant access to nutrients.

In the European Union (EU) and USA, there are currently no legal definitions for the term "biofertilizer," or specific legal provisions defining their characteristics. In the EU, microorganisms (bacteria, viruses, and fungi) are included as possible inputs in the EU Commission Regulation No. 889/2008 on organic production, but only for the biocontrol of pests and diseases. As such, they are thus listed within the legal framework dealing with plant protection products, as biocontrol agents. Similarly, the US National Organic Program foresees only the possibility of using biological organisms for plant protection.

The Government of India vide Gazette notification dated April 10, 2008, incorporated biofertilizers and organic manures in Fertilizer (Control) Order (FCO) defining necessary requirement for registration, standards, procedures, and testing protocols. After notification of Schedule III and IV, wherein biofertilizers and organic manures were included under the ambit of FCO, subsequent amendments have been notified vide Extra Ordinary Gazette Notification of Ministry of Agriculture on November 3, 2009. As per Indian legislations as mentioned in FCO, issued in April 2008, "Biofertilizer means the product containing carrier base (solid or liquid) living microorganisms which are agriculturally useful in terms of nitrogen fixation, phosphorus solubilization or nutrient mobilization to increase the productivity of the soil and/or crop."

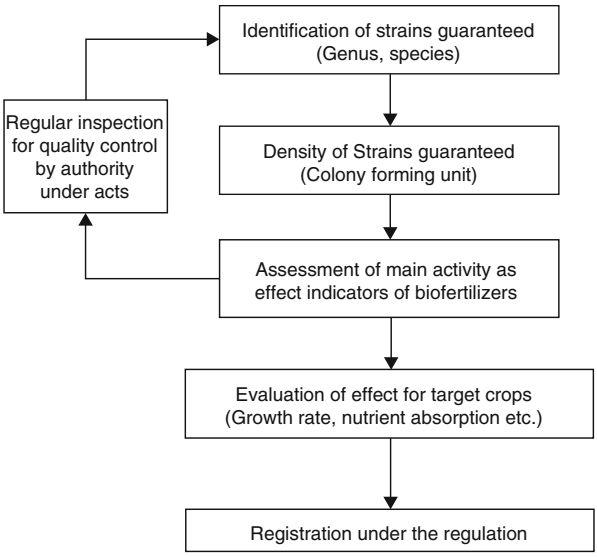

Fig. 8.1 Procedure of biofertilizer quality control

8.4 Quality Control of Biofertilizers

To make any product acceptable, quality control is very essential. It is important to set control plots that do not contain available microorganisms, but whose other compositions are the same as the final microbial products. Also it is highly desirable that the biofertilizer manifests the major effects for the quality management of the final biofertilizer products. The major effects are used as indicators for the biofertilizer. Also, the effects are included as guaranteed activities of the biofertilizer implying that the effects of microbial products have to originate from the guaranteed microorganisms. It is essential to evaluate precisely the functions under the given usage manifested by the applicant (Fig. 8.1).

8.5 Legal Quality of Biofertilizers

High-quality formulation of biofertilizers refers to high density of viable spores/cells/propagules of the desired microorganisms with a suitable inert carrier (Herridge et al. 2008). In short high-quality formulation of biofertilizers should essentially reflect the vital parameters as advertised in labeling. Due to unavailability of universal guidelines for commercial production of biofertilizers, each nation has set their specific parameters for the same. The most vital information to be displayed on the product label must include CFU/cell number, date of manufacture and expiry, batch number, and method of use. Some countries where the biofertilizer industry has been strongly developed have already enacted some regulations. In India and China,

the quality of biofertilizer is based on eight essential parameters, viz., number of viable cells, pH, contamination, granule size, carbon and moisture content, appearance, and date of expiry (Suh et al. 2006).

8.6 Indian Biofertilizer Regulations as Role Model for Asian Countries

India has a strong legal framework related to biofertilizer production. The Ministry of Agriculture and Farmers Welfare, Government of India, issued an order in 2006 that brought biofertilizers under the Essential Commodities Act of 1955 and within the order for the control of fertilizers of 1985. This was later amended in 2009, 2010, and 2015 which brought arbuscular mycorrhiza, *Acetobacter*, potash mobilize, zinc solubilizer liquid, and carrier-based formulations under its preview of FCO (1985). The term biofertilizer is legally defined as "the product containing carrier based (solid or liquid) living microorganisms which are agriculturally useful in terms of nitrogen fixation, phosphorus solubilization or nutrient mobilization, to increase the productivity of the soil and/or crop." The term is also covered under the broad definition of fertilizers, which "means any substance used or intended to be used as a fertilizer of the soil and/or crop." The registration requirements, sampling procedures, scale of sampling, and testing procedures along with other procedures and requirements are clearly defined as:

8.6.1 Certificate of Manufacturing

- As per clause 13, subclause (1) (b) and (c), no person shall manufacture any biofertilizer/organic fertilizer, unless it conforms to the standards set out in the Part A of Schedule III and Schedule IV, respectively.
- Certificate of manufacture has to be obtained from registering authority under clause 14, subclause (3) with requisite fee under clause 36.
- As per clause 21, subclause (ii) (a), every container of Biofertilizer/Organic fertilizer has to be labeled as biofertilizer/organic fertilizers.

8.6.2 Other Required Specifications

8.6.2.1 Packing

Biofertilizer shall be packed in suitable plastic bags/packs, thickness of which shall not be less than 75–100 μm or in suitable plastic bottles.

8.6.2.2 Marking

Each polyethylene pack shall be marked legibly and indelibly with the following information:

(a) Name of the product.
(b) Name and address of the manufacturer.
(c) Crop(s) for which intended.
(d) Type of the carrier used.
(e) Batch number.
(f) Date of manufacture.
(g) "Expiry date which shall not be more than 6 months from the date of manufacture in case of carrier based, powder/granulated formulation of *Rhizobium; Azotobacter; Azospirillium* and PSB, KSB, ZSB biofertilizers and liquid based Biofertilizers, while it shall not be less than 12 months from the date of manufacture in case of liquid based *Azotobacter; Azospirillium* and PSB biofertilizers."
(h) Net mass in kg/gram and area meant for.
(i) Storage instructions worded as "STORE IN COOL PLACE AWAY FROM DIRECT SUN LIGHT AND HEAT."
(j) Any other information required under the Standards of Weight and Measures (Packaged Commodities) Rule (1977).

- Items at S. No. (c),(f), and (g) shall be printed with colored ink on the background.
- Directions for use of biofertilizer shall be printed briefly on the packets.

8.6.3 Procedure for Sampling of Biofertilizers

(a) In drawing, preparing, and handling the samples, the following precautions and directions should be observed.
(b) Sampling shall be carried out by a trained and experienced person as it is essential that the samples should be representative of the lot to be examined.
(c) Samples in their original unopened packets should be drawn and sent to the laboratory to prevent possible contamination of the samples during handling and help in revealing the true condition of the material.
(d) Intact packets shall be drawn from a protected place not exposed to dampness, air, light, dust, or soot.

8.6.4 Scale of Sampling

(a) Lot – All units (containers in a single consignment of type of material belonging to the same batch of manufacture) shall constitute a lot. If a consignment consists of different batches, the container of the same batch shall be separated and shall constitute a separate lot.
(b) Batch – Inoculants prepared from a batch fermentor or a group of flask (container) constitutes a batch.

(c) For ascertaining conformity of the material to the requirements of the specification, samples shall be tested from each lot separately.
(d) The number of packets to be selected from a lot shall depend on the size of the lot and these packets shall be selected at random and in order to ensure the randomness of selection procedure.

8.6.5 Drawl of Samples

(a) The inspector shall take three packets as samples from the same batch. Each sample constitutes a test sample.
(b) The samples should be sealed with the inspector's seal after putting inside FORM P. Identifiable details such as sample number's code number or any other details which enable its identification shall be marked on the cloth bags.
(c) Out of the three samples collected, one sample so sealed shall be sent to in-charge laboratory notified by the state government under clause 29 or to NCOF/RCOFs. Another sample shall be given to the manufacturer, or importer, or dealer as the case may be. The third sample shall be sent by the inspector to his next higher authority for keeping in safe custody. Any of the latter two samples shall be sent for referee analysis under subclause (2) of clause 29B of FCO.
(d) The number of samples to be drawn from the lot is as under:

Lot/batch	No. of samples to be drawn
Up to 5000 packets	03
5000–10,000 packets	04
More than 10,000 packets	05

8.6.6 Notified Testing Laboratories

As per clause 2a, subclause (1) (1A) and (1B), samples of biofertilizers/organic fertilizers are to be tested in National Centre of Organic Farming (NCOF) and Regional Centre of Organic Farming (RCOF), Bengaluru, Bhubaneswar, Imphal, Jabalpur, Nagpur, and Panchkula or other notified state laboratories.

8.6.7 Eligibility of Organic Fertilizer/Biofertilizer Inspectors

(a) Organic fertilizer/biofertilizer inspectors are persons notified under FCO, who is delegated the powers of drawl of samples of organic fertilizers and biofertilizers, as per the procedure laid down in the FCO.
(b) Inspectors of biofertilizer and organic fertilizer are appointed under clause 27B and should possessing the following qualifications:

(I) Graduate in Agriculture or Science with Chemistry/Microbiology as one of the subject.
(II) Trained or experienced in the field of quality control of biofertilizers/organic fertilizers.

8.7 Quality Specification of Biofertilizers as per Indian FCO

8.7.1 *Rhizobium* Biofertilizer

(i)	**Base**	Carrier based* or liquid based
(ii)	**Viable cell count**	CFU minimum 5×10^7 cell/g of carrier material or 1×10^8 cell/ml of liquid material
(iii)	**Contamination level**	No contamination at 10^5 dilution
(iv)	**pH**	6.5–7.5
(v)	**Particle size in case of carrier-based material**	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	**Moisture percent by weight, maximum in case of carrier based**	30–40 %
(vii)	**Efficiency character**	Should show effective nodulation on all the species listed on the packet

***Type of carrier:** The carrier materials such as peat, lignite, peat soil, humus, wood charcoal, or similar material favoring growth of organism

8.7.2 *Azotobacter* Biofertilizer

(i)	**Base**	Carrier based* or liquid based
(ii)	**Viable cell count**	CFU minimum 5×10^7 cell/g of carrier material or 1×10^8 cell/ml of liquid material
(iii)	**Contamination level**	No contamination at 10^5 dilution
(iv)	**pH**	6.5–7.5
(v)	**Particle size in case of carrier-based material**	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	**Moisture percent by weight, maximum in case of carrier based**	30–40 %
(vii)	**Efficiency character**	The strain should be capable of fixing at least 10 mg of nitrogen g^{-1} of sucrose consumed

***Type of carrier:** The carrier material such as peat, lignite, peat soil, humus, wood charcoal, or similar material favoring growth of the organism

8.7.3 *Azospirillum* Biofertilizer

(i)	**Base**	Carrier based* or liquid based
(ii)	**Viable cell count**	CFU minimum 5×10^7 cell/g of carrier material or 1×10^8 cell/ml of liquid material
(iii)	**Contamination level**	No contamination at 10^5 dilution
(iv)	**pH**	6.5–7.5
(v)	**Particle size in case of carrier-based material**	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	**Moisture percent by weight, maximum in case of carrier based**	30–40 %
(vii)	**Efficiency character**	Formation of white pellicle in semisolid N-free bromothymol blue media

***Type of carrier:** The carrier material such as peat, lignite, peat soil, humus, wood charcoal, or similar material favoring growth of the organism

8.7.4 *Acetobacter* Biofertilizer

(i)	**Base**	Carrier based* in form of moist/dry powder or granules or liquid based
(ii)	**Viable cell count**	CFU minimum 5×10^7 cell/g of carrier material or 1×10^8 cell/ml of liquid material
(iii)	**Contamination level**	No contamination at 10^5 dilution
(iv)	**pH**	5.5–6.0 for moist/dry powder, granulated or carrier based and 3.5–6.0 for liquid based
(v)	**Particle size in case of carrier-based material**	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	**Moisture percent by weight, maximum in case of carrier based**	30–40 %
(vii)	**Efficiency character**	Formulation of yellowish pellicle in semisolid N tree medium

***Type of carrier:** The carrier material such as peat, lignite, peat soil, humus, wood charcoal, or similar material favoring growth of the organism

8.7.5 Mycorrhizal Biofertilizers (AM – Biofertilizer)

(i)	**Form/base**	Fine powder/tablets/granules/root biomass mixed with growing substrate
(ii)	**Particle size for carrier based**	90 % material should pass through 250 μm IS sieve powder formulations (60 BSS)
(iii)	**Moisture content percent maximum**	8–12 %

(iv)	**pH**	6.0–7.5
(v)	**Total viable propagules/gram of product, minimum**	100/g of finished product
(vi)	**Infectivity potential**	80 infection points in test roots/gram of mycorrhizal inoculum used
(vii)	**Tolerance limit**	The viable propagules shall not be less than 80

8.7.6 Phosphate Solubilising Biofertilizer

(i)	**Base**	Carrier based or liquid based
(ii)	**Viable cell count**	CFU minimum 5×10^7 cell/g of carrier material or 1×10^8 cell/ml of liquid material
(iii)	**Contamination level**	No contamination at 10^5 dilution
(iv)	**pH**	6.5–7.5
(v)	**Particle size in case of carrier-based material**	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	**Moisture percent by weight, maximum in case of carrier based**	30–40 %
(vii)	**Efficiency character**	The strain should have phosphate solubilizing capacity in the range of minimum 30 % when tested spectrophotometrically. In terms of zone formation, minimum 5.0 mm solubilization zone in prescribed media having at least 3.0 mm thickness

8.7.7 Potassium Mobilising Biofertilizer

(i)	**Base**	Carrier based or liquid based
(ii)	**Viable cell count**	CFU minimum 5×10^7 cell/g of carrier material or 1×10^8 cell/ml of liquid material
(iii)	**Contamination level**	No contamination at 10^5 dilution
(iv)	**pH**	(i). Powder based or granules 6.5–7.5
		(ii). Liquid based 5.0–7.5
(v)	**Particle size in case of carrier-based material**	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	**Moisture percent by weight, maximum in case of carrier based**	30–40 %
(vii)	**Efficiency character**	Minimum 10.0 mm solubilization zone in prescribed media having at least 3.0 mm thickness

8.7.8 Zinc Solubilising Biofertilizer

(i)	**Base**	Carrier based or liquid based
(ii)	**Viable cell count**	CFU minimum 5×10^7 cell/g of carrier material or 1×10^8 cell/ml of liquid material
(iii)	**Contamination level**	No contamination at 10^5 dilution
(iv)	**pH**	(i). Powder based or granules 6.5–7.5
		(ii). Liquid based 5.0–7.5
(v)	**Particle size in case of carrier based-material**	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	**Moisture percent by weight, maximum in case of carrier based**	30–40 %
(vii)	**Efficiency character**	Minimum 10.0 mm solubilization zone in prescribed media having at least 3.0 mm thickness

8.7.9 NPK Liquid Consortia Biofertilizers

(i)	**Individual viable count in liquid-based formulation**	CFU minimum in a mixture of any two or more of following microorganisms:
		CFU minimum *Rhizobium* or *Azotobacter*
		or Azospirillum: 1×10^8 ml^{-1}
		CFU minimum PSB: 1×10^8 ml^{-1}
		CFU minimum KSB: 1×10^8 ml^{-1}
(ii)	**Total viable count of all the biofertilizer organisms in the product**	CFU minimum 5×10^8 cells ml^{-1} of liquid-based formulation
(iii)	**Contamination level**	No contamination at any dilution
(iv)	**pH**	5.0–7.0
(v)	**Efficiency character**	
	Rhizobium	Nodulation test positive
	Azotobacter	The strain should be capable of fixing at least 10 mg N fixation/gm of carbon source
	Azospirillum	The strain should be capable of fixing at least 10 mg N fixation/gm of malate applied
	PSB	Minimum 5 mm zone of solubilization zone on PSB media having at least 3 mm thickness
	KSB	Minimum 5 mm zone of solubilization zone on KSB media having at least 3 mm thickness

8.8 Conclusion

Although biofertilizer technology is a cost-effective and sustainable technology, its market is still lagging as compared to synthetic fertilizers. Many factors are responsible for this sluggish growth, but to ensure a sustainable and effective alternative to chemical fertilizer inputs, biofertilizer technology has to play greater role in the future, particularly in Asia, where agriculture is presently overburdened by the high-cost chemical inputs, especially fertilizers. Regulatory bodies and industry have to share the responsibilities to overcome the constraints and offer high-quality biofertilizers to the end users.

It is highly desirable that the biofertilizer manifests the major effects for the quality management of the final products. Therefore, quality regulation of biofertilizers is important to ensure conformity to prescribed standards, product safety, and efficacy. Poor-quality biofertilizers can be expected in the market when the quality control framework is not well defined, resulting in poor field performance. In Asia lack of effective regulation of biofertilizers is among the greatest contributors to low availability and adoption of the products dissimilar to the situation in selected Asian countries. A well-defined regulatory setup together with the periodic monitoring of products in the market is important to ensure product quality in the full commercialization chain for exploiting the full potential of biofertilizers in Asia.

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Part III

Biopesticide and Biofertilizer Regulatory Requirements in South and Southeast Asia

Research, Development and Commercialisation of Agriculturally Important Microorganisms in Malaysia

9

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Abstract

Agriculture is considered as one of the most important economic sector in Malaysia. Intensive agriculture in Malaysia is highly based on usage of agricultural inputs such as fertilisers and pesticides. Both the agriculture and plantation sectors are operated by using imported fertilisers and pesticides. An injudicious usage of these two inputs in agricultural sectors has created many environmental and health issues. A possible solution to avoid this fertiliser and pesticide crisis may rest with groups of microorganisms that have the capacity to provide the nutrients needed and protection against pests for crops. These beneficial microorganism-based products are known as biofertilisers and biopesticide. The current chapter discusses the research, development and commercialisation of agriculturally important microorganisms in Malaysia.

Keywords

Biofertilisers • Biopesticides • Pesticides • Agriculturally important microorganisms

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9.1 Agriculture in Malaysia: Current Status in Fertiliser and Pesticide Usage

Agriculture is considered as one of the most important economic sector in Malaysia. With 5.6 m ha of cultivated area in 2012, this industry plays an important role in ensuring food security, generating export revenue, creating agro-based industries and generating millions of job opportunities for Malaysian (DOA 2015). Realising the importance of this sector, through its Economic Transformation Programme, the Malaysian Government recognised agriculture and plantation sectors as country's National Key Economic Area (NKEA). NKEA is defined as an important driver of economic activities that potentially and directly contributes towards Malaysia's economic growth measureable by the gross national income (GNI) indicator (Pemandu 2011).

Intensive agriculture in Malaysia is highly based on the usage of agricultural inputs such as fertilisers and pesticides. Both the agriculture and plantation sectors are operated by using imported fertilisers and pesticides. In 2013, the amount of fertiliser and pesticide imported were 3.95 million and 122,885 tonnes, respectively (DOA 2016; FAO 2016). The biggest portion of the production cost on agricultural and plantation crops goes to fertiliser and pesticide purchase. Since these agro-inputs are imported, the fluctuating US dollar exchange value will burden both the sectors and increases the production cost. Reduction in production and threat of war in the main oil-producing regions had a deleterious impact on the fertiliser industry and ultimately agricultural production. Depletion of oil reserves, rising prices, environmental issues and the global greenhouse crisis has further affected this industry with tremendous rise in fertiliser and pesticide prices.

An injudicious usage of these two inputs in agricultural sectors has created many environmental and health issues. Phenomenon such as soil hardening, increased soil salinity, low nutrient release capacity and low water holding capacity are very common when chemical fertilisers are used extensively, thereby contributing to poor nutrient use efficiency (NUE). A possible solution to avoid this fertiliser and pesticide crisis may rest with groups of microorganisms that have the capacity to provide the nutrients needed and protection against pests for crops. These beneficial microorganism-based products are known as biofertilisers and biopesticide (Keswani et al. 2013; Bisen et al. 2015; Mishra et al. 2015).

9.2 Integrated Crop Management for Sustainable Agriculture in Malaysia

Integrated crop management (ICM) is a system of crop production which converse and enhances natural resources while producing food on an economically viable and sustainable foundation. It is based on a good understanding of the interactions between biology, environment and land management systems (Chandler et al. 2008). ICM is a sustainable agricultural production system which minimise the dependency on purchased (imported) inputs (such as fertilisers and pesticides) and

to make optimal use of indigenous input sources. Biofertilisers and biopesticides are two of indigenous inputs which were successfully used for partial or full replacement of synthetic fertiliser and pesticides in various crops globally. Based on our own experiences, low dose of chemical fertiliser (1/3rd recommended rate) in combination with biofertiliser had produced same amount of rice yield as recommended dose of chemical fertiliser, suggesting that partial replacement of chemical fertiliser is possible. Biopesticides are natural pest and disease control strategy used in Integrated Pest Management (IPM) and successfully reduced the amount of pesticide application on the field (Allahyari et al. 2016). Although both biofertiliser and biopesticide were used for a long time in crops production, the performance of these inputs are inconsistent. Therefore production of excellent quality biofertilisers and biopesticide products is a prerequisite for product performance and reliability. Research and development on biofertiliser and biopesticides are crucial for innovation of excellent quality biofertiliser and biopesticide products with reliable performance that can be commercialised. In Malaysia, the Government is encouraging the R&D activities through National Policy on Science, Technology and Innovation (NPSTI).

9.3 National Policy on Science, Technology and Innovation (NPSTI) (2013–2020)

National Policy on Science, Technology and Innovation (NPSTI) was drafted to develop a scientifically advanced nation for socio-economic transformation and inclusive growth towards an innovation technology by 2020 (OECD 2014). This policy is very important in providing guidelines and implementing the strategies for the country to achieve its mission of high-income nation status by 2020. One of the strategic thrusts to achieve this ambition is advancing scientific and social research, development and commercialisation (R, D and C). This policy had changed the landscape of the R, D and C in Malaysia where public funding researches must generate technology that benefitted the country and generating income and job opportunities for public by commercialisation. Biofertiliser and biopesticide researches in the country also have to comply with this policy where commercialisation of the innovation was stressed.

9.4 Research, Development and Commercialisation Funding Continuum: From Lab to Market

There are various funding options available for R&D and commercialisation for innovation generation in Malaysia. The funds offered by various agencies are shown in Table 9.1. Among the fund providing agencies, the Ministry of Science, Technology and Innovation (MOSTI) and Ministry of Education (MOE) are the main research funders. Basic research funds (FRGS, TRGS and LRGS) are open for implementation of activities such as theory development, concept and new ideas

Table 9.1 R, D and C grants offered by various agencies in Malaysia

Funding agency	Grant	Research/commercialisation
Ministry of Education	Fundamental Research Grant Scheme (FRGS)	Basic research
	Transdisciplinary Research Grant Scheme (TRGS)	Basic research
	Long Run Grant Scheme (LRGS)	Basic research
	Prototype Research Grant Scheme (PRGS)	Pre-commercialisation
	Knowledge Transfer Programme (KTP)	Pre-commercialisation
Ministry of Science, Technology and Innovation	Science fund	Applied research
	Techno fund	Pre-commercialisation
	Inno Fund (for individual and microbusinesses)	Applied research
	Biotech commercialisation fund	Pre-commercialisation
	Flagship fund	Commercialisation
		Applied research/ pre-commercialisation
Ministry of Agriculture and Agro-Based industry	NKEA agriculture project fund	Pre-commercialisation
		Commercialisation
Cradle Fund Sdn. Bhd., Ministry of Finance	Cradle fund	Commercialisation

towards knowledge development followed by applied study based on finding of basic studies (Science Fund) which is useful for development of innovation and technologies that can contribute to economy and human well-being. Any potential findings in applied studies can be further developed for commercial products/technologies through pre-commercialisation. Pre-commercialisation is linking between the basic/applied researches with technology commercialisation. There are various funds available for pre-commercialisation such as PRGS, KTP, Techno Fund, Inno Fund and NKEA agriculture project fund. Pre-commercialisation funds can be used for prototype development, pilot plant or commercialisation-ready prototype. The prototype and pilot plant can be commercialised using cradle fund, biotech commercialisation fund and NKEA agriculture project fund. There are additional funds for pre-commercialisation and commercialisation activities suggesting that Malaysian Government is committed for generation of new technologies which contributed for economic development and human welfare.

Fund is also available from various international agencies for research. Private sector also contributes for research by providing fund where the researches were tailored for the company's needs, among them are contract research (research cost fully sponsored by company) and collaborative research (research cost shared by company and research entities).

9.5 Biofertiliser Research in Malaysia

Biofertilisers are substances containing living microorganisms which, when applied to seeds, plant surface, or soil, colonises the rhizosphere or the interior of plant and promotes growth by increasing the supply or availability of primary nutrient to the host plant (Vessey 2003). Biofertilisers promote the plant growth by supplying nutrients through atmospheric nitrogen fixation, phosphorus solubilisation and mobilisation, potassium solubilisation and chelation of trace elements such as iron (Fe^{3+}). Application of biofertilisers with minimal level of chemical nutrient input will attribute to better harvest.

Although there are plenty of good testimonies about the performance of biofertiliser in controlled environments in Malaysia, its performance in the field was inconsistent. Therefore, more intensive research is needed to develop biofertiliser products with excellent and reliable quality.

In the past decades, at least 45 research projects were conducted by universities and public research institute (PRIs) in Malaysia (Table 9.2). The main bacterial genera studied for biofertiliser research are *Bacillus*, *Klebsiella*, *Pseudomonas*,

Table 9.2 Biofertiliser researches conducted in Malaysian PRIs and universities

Institution	Microorganism	Field of study
MARDI	*Bacillus* spp., *Klebsiella* spp., *Pseudomonas aeruginosa*, *P. fluorescens*, *A. brasilense* Sp245, *Rhizobium leguminosarum bv. trifolii* R4, mycorrhiza	PGPR survival
		P-solubilisation
		K solubilisation, microbial activity at community level, physiology, PGPR counting, whole-genome expression, quality control of biofertiliser products
Malaysian Putra University	*Bacillus* spp., *Stenotrophomonas*	Efficacy test
	Burkholderia spp., *Sphingomonas* spp., *Azospirillum brasilense* Sp7, *Bacillus sphaericus* UPMB10, mycorrhiza	Plant growth promotion, root colonisation pattern
		Bioprospection of PGPR, N-fixation
		P- solubilisation
Malaysian National University	*Sphingomonas paucimobilis*, *Arthrobacter globiformis*, *Bacillus cereus*, *Bacillus pumilus*, *Rhizobium rhizogenes*, *Rhizobium radiobacter*	Bioremediation
Malaysian Nuclear Agency	Phosphate-solubilising bacteria	Plant growth promotion, quality control of biofertiliser products, solid media sterilisation using gamma irradiation
	Mycorrhiza	
Malaya University	*Pseudomonas* sp.	Genomic
	Planococcus rifietoensis M8T	
Universiti Malaysia Sarawak	*Bacillus* spp.	Plant growth promotion

Azospirillum, *Rhizobium*, *Stenotrophomonas*, *Burkholderia*, *Sphingomonas*, *Arthrobacter* and mycorrhizal fungi. Among them, the most studied microorganisms are *Bacillus* and mycorrhiza. The main crops studied for biofertiliser studies were oil palm, rice, fruits and vegetables. Among them oil palm and rice are prominent, since they are planted in large cultivated hectare. The main area of focus was biofertiliser strain bioprospection, N-fixation, P and K solubilisation, root colonisation, biofertiliser efficacy testing and bioremediation. However, another important research area in biofertilisers which was less investigated was bioremediation, which indicating the usage of biofertilisers for bioremediation is getting less attention in Malaysia. Most of soil bioremediation works conducted in Malaysia are mainly based on organic fertiliser and other synthetic chelating.

There is huge number of industry-sponsored biofertiliser researches conducted by universities and PRIs for testing the efficacy of their imported biofertiliser products. Product efficacy trials are crucial for testing the suitability and survival of industrial products in native conditions.

9.6 Biopesticide Research in Malaysia

Biopesticides are pest control tool derived from biological resources including several types of pest management strategies through predatory, parasitic and antagonistic activities (Soleder and Lacey 2013; Glare et al. 2012). Biopesticide are naturally occurring or derived substance or an organism which controls pests by non-toxic means. There are wide spectrums of potential products that can be classified as biopesticide:

(i) Microbial pesticide and entomopathogens – containing microorganisms such as virus, bacteria, fungus and nematodes
(ii) Plant-incorporated protectant – genetically modified plants that posses genes which control pest and diseases
(iii) Biochemical protectant – containing pheromones, plant extracts and natural growth regulators
(iv) Natural enemies

In this chapter, only the microbial biopesticide have been discussed due to their growing significance in plant disease management in Malaysia.

Although biopesticide has been used for a long time in Malaysian agriculture, their performance was less satisfactory; thus, farmers have negative perceptions. The main concern for farmers is that they desire very quick effects of biopesticide equivalent to chemical pesticides. In the past decade, at least 96 research projects on biopesticides were executed by universities and PRIs (Table 9.3). The main microorganism types used for biopesticide researches in Malaysia were fungi (*Metarhizium*, *Beauveria*, *Trichoderma* and *Paecilomyces*), bacteria (*Bacillus* and *Pseudomonas*) and viruses (*Nucleopolyhedrovirus* and bacteriophage). The main research focus was bioprospection of microbial biopesticide, biopesticide

Table 9.3 Biopesticide projects conducted in Malaysian PRIs and universities

Institution	Biocontrol microorganism	Targeted pathogen/disease	Crop protected
MARDI	*Trichoderma* sp.	Stem end rot	Mango
		(*Botryodiplodia theobromae*)	
	Bacillus sp. 289	*R. solani*	Rice
	Trichoderma sp.	Anthracnose	Chilli
	Sordariomycetes sp.	*Colletotrichum gloeosporioides* *Colletotrichum capsici*	
	Stagonosporopsis sp.		
	Streptomyces sp. S2	Anthracnose	Chilli
	Bacteriophage	Papaya dieback (*E. mallotivora*)	Papaya
	Bacillus spp.	Papaya dieback (*E. mallotivora*)	Papaya
	Steinernema sp.	Insects	
	Bacillus spp.	*Fusarium* wilt	Banana
		Fusarium oxysporum f. sp. *cubense*	
	Trichoderma sp.	Fusarium wilt	Banana
		Fusarium oxysporum f. sp. *cubense*	
	Unidentified	*Rice diseases caused by:*	Rice
		X. oryzae, *X. campestris*, *Fusarium moniliforme*, *Pyricularia oryzae*, *Helminthosporium oryzae*, *R. solani*, *Sarocladium oryzae*	
	Bacillus 23S	Papaya dieback	Papaya
		(*E. mallotivora*)	
	Unidentified bacteria	Papaya dieback	Papaya
		(*E. mallotivora*)	
	Unidentified bacteria	Bacterial heart rot	Pineapple
		(*E. chrysanthemi*)	
	Unidentified bacteria	Durian canker (*Phytophthora palmivora*)	Durian
	Trichoderma asperellum	(*Colletotrichum gloeosporioides*)	Mango
	Metarhizium spp.	Fruit fly	Papaya
		(*Bactrocera papayae*)	
	Exserohilum monoceras	Weed	Rice
		Echinochloa sp.	
Universiti Putra Malaysia	*Pseudomonas aeruginosa* (UPMP3), *Burkholderia cepacia* (UPMB3), *Serratia marcescens* (UPMS3)	Anthracnose	Chilli
		Colletotrichum capsici, shoot blight (*Choanephora cucurbitarum* L.), and foot and collar rot (*Sclerotium rolfsii* Sacc) are the most important diseases associated with chilli production	

(continued)

Table 9.3 (continued)

Institution	Biocontrol microorganism	Targeted pathogen/disease	Crop protected
	B. subtilis B34	Anthracnose	Papaya
		Colletotrichum gloeosporioides	
	Diazotrophic bacteria	Seed-borne fungal pathogen	Kenaf *Lasiodiplodia theobromae*, *Diaporthe* sp., *Fusarium* sp.
	Paecilomyces lilacinus	–	Termite
	Unknown bacteria	*Colletotrichum gloeosporioides*	Papaya
	Bacillus amyloliquefaciens	Fusarium wilt	Rock melon
	Alcaligenes faecalis	(*F. oxysporum f.* sp. *melonis*)	
	Bacillus subtilis A2	Soft rot	Vegetables
		Pectobacterium spp.	
	Nucleopolyhedrovirus	*Spodoptera litura*	Vegetables
	Nucleopolyhedrovirus	*Spodoptera litura*	Vegetables
	Metarhizium anisopliae	Termite	Plantation crops
	Nucleopolyhedrovirus	*Spodoptera litura*	Vegetables
	Arbuscular mycorrhizal	Basal stem rot	Sweet corn
		(*Rhizoctonia solani*)	
	P. fluorescent	Rice sheath blight	Rice
	P. aeruginosa		
	P. asplenii		
	Nucleopolyhedrovirus	*Spodoptera litura*	Vegetables
	T. harzianum, *T. hamatum*, *T. koningii*	Fusarium ear rot	Corn
		(*Fusarium* spp.)	
FELDA	*Metarhizium anisopliae*	*Oryctes rhinoceros*	Oil palm
FRIM	*Metarhizium anisopliae* and *Beauveria bassiana*	Tiger moth	Forestry
Lembaga Koko Malaysia	*Beauveria bassiana*	Cocoa pod borer (*Conopomorpha cramerella*)	Cocoa
Malaysian National University	*Bacillus subtilis*	Sheath blight disease	Rice
	Trichoderma spp.	(*R. solani*)	
MARA Technology University	*Actinomycetes* sp.	Root disease	*Acacia mangium*
	P. aeruginosa		*Azadirachta*
	Trichoderma sp.		*Tectona grandis*, rubber
MPOB	*Basidiomycetes fungi*	Basal stem rot	Oil palm
		(*G. boninense*)	

(continued)

Table 9.3 (continued)

Institution	Biocontrol microorganism	Targeted pathogen/disease	Crop protected
	Trichoderma spp.	Basal stem rot (*G. boninense*)	Oil palm
UM	*Streptomyces malaysiensis* NBRC16446,	*Fusarium semitectum*, *F. oxysporum*, *F. decemcellulare*	Dragon fruit
	Streptomyces cavourensis subsp. *cavourensis* NBRC13026		
	Streptomyces sanyensis FJ261968446		
UM	*Streptomyces rochei* strain A1, *Streptomyces rubrogriseus*, *Streptomyces purpeofuscus*	*Colletotrichum capsici*, *C. acutatum*, *C. gloeosporioides*	Chilli
CABI Malaysia	*M. anisopliae Beauveria bassiana*	Tiger moth, bagworm	Oil palm

formulation, mass production of biopesticide products, field application technique and efficacy testing in open field.

There were a significant number of private-sponsored biopesticide projects conducted in collaboration with by universities and PRIs for testing the efficacy of their improved products. These researches are crucial to testing the suitability and survival of these products in native conditions before the products can be marketed.

9.7 Protection of Intellectual Property Right (IPR) on Biofertilisers and Biopesticide

The intellectual properties generated in Malaysia and abroad can be protected by filing intellectual property rights (IPR) to Intellectual Property Corporation of Malaysia (MyIPO). The IPRs filed with MyIPO are regulated and protected by Patents Act 1983 and Patents Regulation 1986.

The IPRs filed on biofertiliser and biopesticides are shown in Table 9.4. Forty intellectual property rights (IPRs) on biofertilisers and biopesticides were filed, where 10 and 30 IPRs were for biofertilisers and biopesticides, respectively. Only 3 IPRs were granted for biofertliser and 4 for biopesticide. The percentage of IPRs filed by PRIs and universities for biofertiliser and biopesticides are 20–47 %, respectively. The remaining IPRs in these both categories are owned by private companies. Relatively more researches were conducted on biopesticide than biofertilisers, which explains why more IPRs were filed for biopesticide. The imported biopesticide products were protected by IPRs with MyIPO before they were marketed.

Table 9.4 Intellectual property rights on biofertilisers and biopesticides

	Biofertilisers		Biopesticide	
Intellectual property rights	Filed	Granted	Filed	Granted
Patent	9	3	30	4
Trademark	1	0	0	0
Total	10	3	30	4

There was significant innovation in the field of biofertilisers and biopesticides which are kept as trade secret by industry and not filed to MyIPO. Therefore, the number of innovations generated for both technologies is supposed to be higher than that shown in the Table 9.4.

9.8 Commercialisation of Biofertilisers and Biopesticide

9.8.1 MARDI Technology Transfer System as a Model of Technology Transfer

National Policy on Science, Technology and Innovation (NPSTI) emphasise on the commercialisation of technology for wealth generation and social well-being of the Malaysian population. MARDI as PRI prioritise technology and IPR commercialisation to fulfil the nation's agenda based on NPSTI.

The technology commercialisation by MARDI is conducted by Technology Commercialisation Office (TCO). The process flow of technology commercialisation by MARDI is illustrated in Fig. 9.1. Any technology generated by MARDI including the biofertilisers and biopesticides needs to go through this process flow (Dardak and Adham 2014).

Generally, R&D outcomes are transferred to extension agencies such as Department of Agriculture (DOA) or Farmers' Organization Authority (FOA) and private companies. These agencies will transfer the technologies to stakeholders such as farmers, farmers association, entrepreneurs and public agencies.

Technology transfer in MARDI involves two process flows (Fig. 9.1) as listed below:

(i) Technology is transferred from MARDI to extension system before it finally reaches the technology users.
(ii) Technology is directly transferred from MARDI to technology users.

The process of technology transfer in MARDI divided into five stages:

(i) The development of new technology

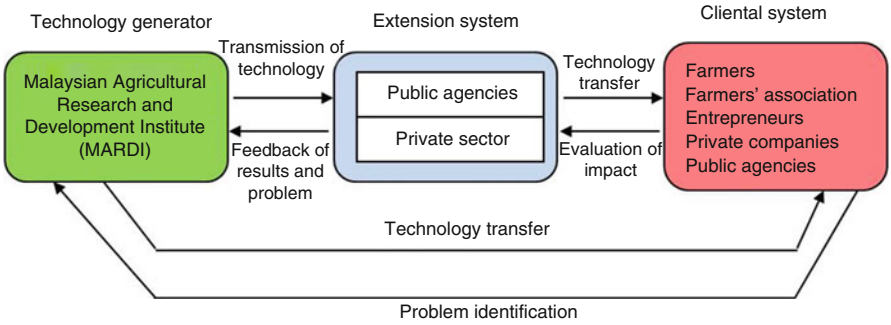

Fig. 9.1 Process flow of technology commercialisation at MARDI

(ii) The evaluation of the technology and approval for commercialisation by MARDI committee for technology management
(iii) Pre-commercialisation
(iv) Commercialisation
(v) Post-commercialisation.

The technology is evaluated for its commercial potential by TCO based on the criteria such as proof of concept, novelty of innovation, technology competitiveness and potential market. Once the technology is transferred to the end user, TCO evaluates the impact of technology after certain period of time. Feedbacks on the results and problems of the technology are evaluated and the corrective action is taken immediately. Generally, other agencies in Malaysia also implement the same process with minor changes or modifications.

9.8.2 Methods of Technology Commercialisation in MARDI

The technologies generated by MARDI are commercialised using eight methods. The methods are listed below:

(i) Licencing
(ii) Consultation
(iii) Contract manufacturing (OEM)
(iv) Outright sale
(v) Joint venture
(vi) Spin-off companies
(vii) Leasing
(viii) Profit sharing

Among them, (i–iv) are the most common methods used for technology commercialisation in MARDI. At least, three biofertilisers and biopesticide technologies were commercialised using licencing method.

Table 9.5 Technology transfer of biofertilisers and biopesticide innovations in Malaysia

Product	Product description	Active microorganism	Technology developer	Technology recipient
3 in 1 N-Fixer	Biochemical fertiliser with effective microorganisms	*Klebsiella* sp.	MARDI	All Cosmos Industries Sdn. Bhd.
Bacteriophage	Biochemical fertiliser with bacteriophage	Bacteriophage P631		
Gano EF	Biochemical fertiliser with biopesticide	*Hendersonia* fungus with effective microorganisms	MPOB	All Cosmos Industries Sdn. Bhd.
MPOB F4	Biochemical fertiliser with effective microorganisms	Unknown		
MycoGold	Mycorrhizal biofertilisers	Mycorrhiza	UPM	MyAgri Sdn. Bhd.
Bacto10	Bacterial biofertilisers	*Bacillus* sp.	UPM	PhytoGold Sdn. Bhd.
Bioliquefert	Bacterial biofertilisers	*Bacillus* sp.	Nuclear Malaysia	

9.8.3 Technology Transfer of Biofertiliser and Biopesticide Innovations

Various biofertilisers, biopesticide and biofertilisers-biopesticide combination technologies were transferred to private companies (Table 9.5). Seven microbial-based products were transferred to four private companies. Among them, All Cosmos Industries Sdn. Bhd. received most of the technologies that were transferred.

9.9 Biofertilisers and Biopesticide Products in Malaysian Market

Biofertilisers and biopesticides marketed in Malaysia are either manufactured locally or imported. Currently, there are 44 biofertiliser products in the markets sold by 22 suppliers (Table 9.6). For microbial-based biopesticide, there are 19 products marketed by six suppliers (Table 9.7).

Biofertilisers sold in this country can be categorised as bacterial and fungal based. Mycorrhiza is the main fungal-based biofertilisers available in the market. The bacterial-based biofertilisers contain various plant growth-promoting bacteria, mainly from the *Bacillus* genus. The bacterial-based biofertilisers are formulated using either single strain or multi-strains.

Microbial biopesticide sold in this country can be categorised as bacterial and fungal based too. *Metarhizium* is the main fungal-based biopesticide available in the market. The popular bacterial-based biopesticide contains *B. thuringiensis*.

The types of formulations common for biofertilisers and biopesticide marketed in the country are in liquid, solid and wettable solid (freeze-dried products) forms. The bacterial-based biofertilisers and biopesticide are mainly in the liquid and wettable solid forms. Both fungal-based biofertilisers and biopesticide product are available in solid form. The number of wettable freeze-dried microbial products is also increasing as it is convenient, easy and cheaper for transportation.

There are also solid biochemical fertiliser cum biopesticide which are formulated by combining inorganic nutrients, organic fertiliser and biofertilisers and biopesticides. All Cosmos Industries Sdn. Bhd. are the prominent biochemical fertiliser manufacturer in the country where the production technology was transferred to Malaysia from Taiwan (Real Strong Max 99 and Max K). The biopesticide-based biochemical fertilisers are Real Strong Gano EF. There are also biochemical fertiliser products (Real Strong MPOB F4 and Gano EF) which were formulated by technologies transferred to All Cosmos Industries Sdn. Bhd. by the local PRIs.

The biofertilisers and biopesticides available in the Malaysian market are mainly for plantation crop (oil palm and rubber), rice, fruit and vegetable production. The main target of the product suppliers are on plantation crops and rice.

Table 9.6 Biofertilisers sold in Malaysia market

Supplier	Products
All Cosmos Industries Sdn. Bhd.	Real Strong brand: MPOB F4, Gano EF, Max 99, Max K
Agricultural Chemicals (M) Sdn. Bhd.	Deliver
JQ Biotech Sdn. Bhd.	Meta-Grow, Meta-OP
Bio-S	Bio-S organic Fertilizer
Starag Corporation Sdn. Bhd.	Phosphate-solubilising bacteria, mycorrhizal fungi
Osaka Marketing (M) Sdn. Bhd.	Master Bio Fertilizer
EMRO Malaysia Sdn. Bhd.	EM 1
EQ Resources	EQ turbo Solution
Chang Chun Chan Enterprises (M) Sdn. Bhd.	Japanese Organic Fertilizer
MyAgri Sdn. Bhd.	MycoGold, Agricare bio-organic, Agricare Organic-N
Biotrack Sdn. Bhd.	RhizaGold
PhytoGold Sdn. Bhd.	Bacto 10
IBG Manufacturing Sdn. Bhd.	IBG Oil Palm Biofertilizer, IBG Multi Purpose Biofertilizer
LKB Biofertilizer	Warisan Microbiological Fertilizer
Green Plant Organic Fertilizer Sdn. Bhd.	Living Organic Fertilizer
Starag Corporation Sdn. Bhd.	Fert-Root, Fert-P, Vigor- BS, Vigor-BM
PR Biotech Marketing Ent.	Bomo Super

Table 9.7 Microbial-based biopesticide sold in Malaysia market

Supplier	Products	Target pathogen/pest
All Cosmos Industries Sdn. Bhd.	Real Strong brand: Gano EF	*Ganoderma boninense*
JQ Biotech Sdn. Bhd.	Meta BT, Meta-Guard	Lepidoptera, disease suppression
Felda Agricultural Services Sdn. Bhd.	ORY-X, Terakil 1, Teracon 1, Lepcon, Bafog, Ecobac	Bagworm
B CUBE Enviro Sdn. Bhd.	BREVA	Coleoptera
Starag Corporation Sdn. Bhd.	Vigor-MA	Lepidoptera, Coleoptera, Canalielates, Orthoptera
Halex (M) Sdn. Bhd.	*B. thuringiensis* var. *kurstaki*	Lepidoptera
	B. thuringiensis subsp. *kurstaki*	
	B. thuringiensis subsp. *aizawai*	
	B. thuringiensis subsp. *israelensis* serotype H-14	
	B. thuringiensis var. *israelensis* H-14	

9.10 Permit Requirement for Biofertilisers and Biopesticide Marketing

Biofertilisers and microbial-based biopesticides available in the country are produced locally or imported. There is no permit required for marketing of locally manufactured biofertiliser and biopesticide products. However, import permit is compulsory for marketing of imported products. There are two types of permits required for microbial product importation and marketing:

(i) Permit to import sample – for sample importation for related authority analysis (small consignment up to 2 kg or 2 l)
(ii) Import permit – for bulk consignment importation for marketing

9.10.1 Application of Permit to Import Sample

Permit to import sample can be applied by using an off-module (non-electronic) application.

Off-module application can be applied for the reasons given below:

(i) Personal materials and sample owner (personal effects)
(ii) Department/government agencies
(iii) Participants of exhibitions
(iv) Researcher/students
(v) Materials imported for first time

For this application, applicants have to enclose a verification letter from organiser or related party. Application can be submitted to Crop Protection and Quarantine Section, Department of Agriculture of Malaysia (JPK) by filling the off-module import permit application form (EP-4A Form) and enclose a banker's draft, postal order or money order valued RM 15.00 and paid to the Director General of Agriculture. Incomplete EP-4A Form will be rejected by the authority. Applicants are requested to apply for the permit 30 days before the importation of microbial product samples.

The information requested in the EP-4A form is as listed below:

1. Importer name and address.
2. Telephone no./fax no.
3. Exporter name and address.
4. Fertiliser/product commercial name.
5. Raw material blends with fertiliser/product (e.g. animal dung, sugarcane waste, paddy husk and others).
6. Microorganism (bacteria, fungus and others scientific name) blends with fertiliser/product. (Please enclose certified letter from manufacturer if the fertiliser does not contain any microorganism).
7. Lab procedure or protocol in the process of microorganism existence in the fertiliser/product.
8. Declaration from responsible authority (government authority) that the fertiliser/product does not contain any ingredients which can cause harmful effect to any plant, livestock, fish, human and environment.
9. NPK content (such as nitrogen 8%, phosphorus 10%, potash 7% – if applicable).
10. Mineral (such as manganese, iron – if applicable).
11. Others (if applicable).
12. Manufacturing process of fertiliser/product (flowchart enclosed).
13. Fertiliser/product form (solid substance/liquid/granule).
14. Country of manufacturer.
15. Other countries using this fertiliser/product.
16. Purpose for using the fertiliser/product (e.g. root growth).
17. Fertiliser/product effect on plants.
18. Fertiliser/product effect on livestock.
19. Fertiliser/product effect on livestock.
20. Fertiliser/product effect on human and environment.

For the materials that are imported in for the first time, the amount of organic fertiliser, microorganisms and materials containing microorganism allowed is 2 l, 2 kg or 5 units per samples (test tubes/ampoules) for analytical purposes. For importing sample, the entry point will only be the Kuala Lumpur International Airport (KLIA). Applicant has to pay RM 340.00 by using the method mentioned above as analysis fee for the samples. Five sets of the biofertilisers and biopesticide samples, each weighing 250 g (powder/solid) or 250 ml (liquid), need to be

submitted during application. Analysis will be conducted by four government agencies, viz. Department of Agriculture, Department of Fisheries, Department of Veterinary Services and Institute of Medical Research, to ensure that the item will not endanger plants, fish, livestock, humans and environment.

The analysis results of the products will be presented to the Committee for Microorganisms Importation (MOBO). The committee consists of representatives from 17 Government agencies which will evaluate the products safety and risk for importation. If there is no objection from the committee member, the product import will be permitted by release of Permit to Import Plants/Soil/Rooting Compost/Growing Media/Beneficial Organism/Organic Fertiliser. If there is objection, the permit will be hold and more information about the products would be requested from the importer, or the committee will request the importer to submit new samples (five sets) for the second analysis. The importer has to bear the cost of RM 340.00. The second analysis results of the products will be presented again to MOBO. If the results are accepted by committee members without any objection, the permit will be released. But, if there is objection, the permit application will be rejected.

9.10.2 Application of Permit to Import Biofertilisers and Biopesticide in Large Quantity for Marketing

Any import and export of materials into Malaysia requires an import/export permit. The permit needs to be applied electronically (ePermit) through an appointed vendor Dagang Net Technologies Sdn. Bhd. (DNT). Importer, exporter, forwarding agent and individual including foreigner have to be registered with Dagang Net for ePermit access. Registration form can be downloaded (www.dagangnet.com) or obtained from DNT branches in the country. A registration fee of RM 500 applied for corporate company and RM 200 for Small and Medium Enterprises (SME). A fee of RM 200 is applied for annual renewal of ePermit.

ePermit application for any import of organic fertilisers, biofertilisers, biopesticides, microorganism and material containing microorganisms is required to import sample as prerequisite for permit application from JPK, Department of Agriculture of Malaysia. Once the ePermit application is registered with DNT, the applicant needs to inform JPK to initiate the online application. The applicant is requested to open a deposit account to enable import/export permit online application using ePermit Deposit Form EP-1. The minimal amount needed for a deposit account opening is RM 150.00.

Before the bulk consignment of biofertilisers and biopesticides arrives, the JPK personnel will visit the warehouse where the products will be stored. If the personnel is satisfied with the warehouse condition for storage, the import permit will be granted. The JPK personnel will also collect the samples from three consecutive bulk consignments of the product arrived and will be analysed again, and the results will be presented to the MOBO committee. If the analysis data shown there has any discrepancies, the import permit will be revoked.

9.11 Future of Biofertilisers and Biopesticide in Malaysia

9.11.1 Farmers' Acceptance and Perception on Biofertilisers and Biopesticide

If compared with 10 years ago, relatively more Malaysian farmers are willing to try biofertilisers and biopesticides for their crop production. The inconsistency in quality and efficacy are fading the farmers' acceptance and confidence on these products. The uncertainty in product efficacy is mainly caused by the lack of stringent quality control during production, transportation and storing of these products. Lack of regulatory control and standard further diminish the quality of these products; thus, the farmers do not have good perception on the products. Therefore, standards and regulation for production of biofertilisers and biopesticides with high-quality products are urgently needed to increase the acceptance of these products.

9.11.2 Biofertiliser Standard

Malaysia does not have any standards for biofertilisers and biopesticide yet. Currently the Fertiliser Technical Committee under the Department of Standards Malaysia had just set-up a working group to draft a Malaysian standard for biofertilisers. Hopefully, in another 2–3 years, Malaysia may have its own standard to regulate the quality of biofertilisers sold in Malaysia.

9.11.3 Encouraging Business Environment for Biofertilisers and Biopesticide

Biofertilisers and biopesticide were two of the focal areas of agricultural biotechnology of Malaysian National Biotechnology Policy. Therefore research, development and commercialisation of these two products have become the priority of the government. Various incentives such as BioNexus status were given to the related companies to commercialise their products. BioNexus is a special status awarded to qualified international and Malaysian biotechnology companies which enjoy fiscal incentives, grants and other guarantees to assist growth. BioNexus status is awarded to qualified companies undertaking value-added biotechnology and/or life sciences activities. To date, there were 11 biofertiliser and biopesticide companies which have been given BioNexus status as recognising the importance of these technologies. The government efforts in encouraging sustainable production system and green technologies also contribute to the expansion of biofertiliser and biopesticide usage among farmers.

9.12 Biofertilisers and Biopesticide Way Forward

Biofertilisers and biopesticide can play an important role in sustainable crop production. They have a huge potential to partially or fully replace the chemical fertiliser and pesticides. The inconsistency in the performance of these both inputs needs to be addressed. The biofertiliser and biopesticide products need to be more competitive, versatile and effective. New types of biofertilisers which can be used for a wide range of crops are urgently needed, while for biopesticide, products active against wide range of pest are needed. Collaboration with private companies is crucial for efficient transfer of technology and commercialisation. More incentives to biofertiliser and biopesticide companies in support of 'green technology' for food safety and societal well-being will encourage private commitment in production and commercialisation of these products.

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Development and Application of Agriculturally Important Microorganisms in India

10

Harikesh Bahadur Singh, Chetan Keswani, Kartikay Bisen, Birinchi Kumar Sarma, and Pranjib Kumar Chakrabarty

Abstract

Significant expansion in agricultural yield brought by green revolution has not been converted into a replica for prolonged agricultural growth. Disproportionate reliance on chemical fertilizers mainly nitrogen and phosphorus and non-judicious use of pesticides have led to water pollution and untenable burden on the agricultural ecosystems. This alarming situation has paved way for augmentation in biofertilizer and biopesticide uses, as commercially available alternative for improving soil quality, and utilization of different resources more effectively for escalating crop production. The coverage of biofertilizer and biopesticide usage in various states of India and its influence on agricultural production has been studied intensively in the current work. The uses of biofertilizers and biopesticides have been promoted actively by both state and central government in India. This chapter comprehensively discusses the current scenario and various regulatory and commercialization challenges associated with the use of biopesticides and biofertilizers. In this chapter few general recommendations have been suggested to enhance the promotion and acceptance of green agriculture in India.

Keywords

Biofertilizer • Biopesticides • Regulation • Pollution

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10.1 Introduction

Sustainable crop production depends largely on soil conditions. Optimum combination of both organic and inorganic components in the soil is imperative for maintaining good soil health. Post-green revolution, the dependence on chemical pesticides and fertilizer application in Indian agriculture has increased manifold. Constant and excessive use of chemical fertilizers leads to devastation of soil biota (Mishra et al. 2015; Bisen et al. 2015; Keswani et al. 2014). In Uttar Pradesh, Punjab, and Haryana, it has reached alarming levels, risking human health and ecological balance. It has therefore become imperative to look for some alternatives which are not only eco-friendly and efficient in enhancing soil fertility but also effective in management of pests and diseases. Biopesticides and biofertilizers have an important role to play in the promotion of sustainable agriculture. These green inputs have significant advantages over the chemical counterparts, and so government agencies like the Ministry of Agriculture and Farmers Welfare, the Department of Biotechnology (DBT), and the Ministry of Science and Technology are actively involved in funding research, development, and marketing of these green inputs. However, despite all these efforts, the acceptance and diffusion of green inputs in India agricultural market is limited. This chapter comprehensively discusses the current scenario and various regulatory and commercialization challenges associated with limited use of biopesticides and biofertilizers. The chapter also suggests few general recommendations or guidelines to enhance promotion and acceptance of green agriculture in India.

10.2 The Indian Market for Biopesticides: Current Status and Regulatory and Commercialization Challenges

10.2.1 Current Scenario

Biopesticides are ecologically acceptable naturally occurring microorganisms used to control pests by nontoxic mechanisms. Depending upon the types of active ingredients involved, biopesticides are categorized into biochemical, plant-incorporated protectants and microbial pesticides. The major advantages of biopesticides in contrast to chemical pesticides are target specificity, environmental safety, efficacy, and biodegradability (Kumar and Singh 2015; Keswani et al. 2013; Bisen et al. 2016). This qualifies biopesticide as a suitable replacement for chemical pesticides.

At global scale, biopesticides comprise only 4 % of the plant protectants; however, the last two decades has seen a steady increase in its growth rate. Worldwide production and distribution of biopesticides has increased manifold since its inception and is further expected to grow with a compound annual growth rate of 20 % (Market and Market 2013). Globally, approximately 1400 biopesticide products are available in market, and the major producers and consumers of biopesticides are the North American Free Trade Agreement (NAFTA) countries (Marrone 2007). A market study reported that the USA, Mexico, and Canada consume about 47 % of the biopesticides sold globally, while Asia with mere 5 % consumption is lagging

Table 10.1 List of representative biopesticides registered in India under section 9(3) of the Insecticides Act, 1968

Biopesticides	Taxus	Formulations	Targets	Trade name
Bacillus thuringiensis subsp. *israelensis*	Bacterium	5.0 % WP, 5.0 % AS	Lepidopteran pests	Tacibio
B. thuringiensis subsp. *kurstaki*		5.0 % WP, 7.5 % WP	Lepidopteran pests	Bio-Dart
Pseudomonas fluorescens		0.5 %, 1.0 % WP	Soilborne diseases	Biomonas
Trichoderma viride	Fungus	1.0 % WP	Soilborne pathogens	Bioderma
Beauveria bassiana		2.15 % WP, 10 % SC or 1.0 %, 1.15 %	Coffee berry borer, diamondback moth, grasshoppers, whiteflies, aphids	Myco-Jaal
Ampelomyces quisqualis		2.0 % WP	Powdery mildew	Bio-Dewcon
Trichoderma harzianum		0.5 %, 1.0 %, 2.0 % WP	Soilborne pathogens	Biozim
Metarhizium anisopliae		1.0 %, 1.5 % WP	Coleoptera, Lepidoptera, termites, mosquitoes, leafhoppers, beetles, grubs	Biomet
Paecilomyces lilacinus		1.0 %	Whitefly	Yorker
Verticillium lecanii		1.15 %	Whitefly, coffee green bug, homopteran pests	Verisoft
Verticillium chlamydosporium		1.0 % WP	Nematodes	
Nuclear polyhedrosis virus of *Helicoverpa armigera*	Virus	0.43 %, 0.5 %, 0.64 %, 2.0 %	*Helicoverpa armigera*	Helicide
Nuclear polyhedrosis virus of *Spodoptera litura*		0.5 %, 2.0 %	*Spodoptera litura*	Spodocide

Note: *WP* wettable powder, *AS* aqueous solution or aqueous suspension, *SC* suspension concentrate

behind (Bailey et al. 2010). The positive impacts of biopesticides have failed to diffuse into the Indian biopesticide market and consequently it is lagging behind, growing at a slow pace. According to a study, India's share of the global biopesticide market was approximately 2.89 % during 2005, which gradually increased to 4.5 % by 2010.

In India, until October 2013, only 14 types of biopesticides with their formulations were registered under the Insecticide Act, 1968 (Table 10.1). Poor technology

is the main hurdle for large-scale industrial production of biopesticides. Besides, there are many regulatory and commercialization constraints for diffusion of biopesticide products in Indian agricultural market.

By 2013, approximately 400 biopesticide active ingredients were registered. In addition, the Ministry of Agriculture and Farmers Welfare is providing necessary guidelines and assistance to as many as 35 commercial companies and 32 integrated pest management (IPM) centers for biopesticide production. To accelerate the production of few screened potential biocontrol agents, the state departments of agriculture and horticulture of Gujarat, Uttar Pradesh, Karnataka, Tamil Nadu, Andhra Pradesh, and Kerala have established numerous advance biocontrol laboratories. Indian Council of Agricultural Research (ICAR) institutions and a few State Agricultural Universities (SAUs) are also involved in microbial pesticide production (Rabindra 2005). The count of biopesticide production units in India have increased to 410, out of which 130 are in the private sector (Singhal 2004; Singh et al. 2012; Desai et al. 2016).

10.2.2 Regulatory Framework and Regulation Constraints for Biopesticides in India

In India, biopesticide is included in the Insecticide Act of 1968, which frames general guidelines with respect to pesticide's bioefficacy and safety to human beings and ecosystem. The Central Insecticides Board & Registration Committee (CIBRC) is the main regulatory body under this Act. CIB as the apex advisory body maintains a strong network of eminent scientists from all disciplines concerned. The RC is involved in granting registrations and licenses to the amateur biopesticide manufacturers. A standard procedure is followed during this entire process. The novel biopesticide formulation is scrutinized by various quality check protocols, and its potential risk associated with human health and ecosystem is properly analyzed. Inclusion of biopesticides in the integrated pest management (IPM) program initiated by the government was the first stepping stone toward the rise of biopesticide products in India. The main government agencies behind the promotion and creation of awareness among farmer's community about biopesticides in India are the Ministry of Agriculture and Farmers Welfare and the Department of Biotechnology (DBT). The Central IPM Centre (Faridabad), the National Centre for IPM (NCIPM) under ICAR, and the Directorate of Biological Control are also among the key players promoting diffusion of biopesticides in agricultural fields (Alam 1994). The DBT funds research in development of biopesticides and also helps in generation of toxicological data for registration purposes. There are also agencies like the National Agricultural Research System (NARS) and the National Board of Accreditation (NBA) whose primary responsibility lies in conducting various standard quality control tests of biopesticides. Besides they are also actively involved in training the officers of the state departments of agriculture in quality control protocols. The state government's role in implementing IPM programs is indispensable. Their IPM programs include purchase and distribution of biopesticides to farmers at affordable

Table 10.2 CIB guidelines/data requirements for registration of biopesticides for minimum infrastructure facilities to be created by the manufacturers of biopesticides

Sl. no.	Particulars
1	Guidelines/data requirements for registration of baculoviruses – nuclear polyhedrosis viruses (NPV) and granulosis viruses (GV) u/s 9(3B) and 9(3) of the Insecticide Act, 1968
1.1	Indian standards – baculoviruses, nuclear polyhedrosis viruses (NPV) and granulosis viruses (GV) specifications
2	Guidelines/data requirements for registration of antagonistic fungi u/s 9(3B) and 9(3) of the Insecticide Act, 1968
2.1	Indian standards – antagonistic fungi specifications
3	Guidelines/data requirements for registration of entomogenous fungi u/s 9(3B) and 9(3) of the Insecticide Act, 1968
3.1	Indian standards – entomopathogenic fungi – specifications
4	Guidelines/data requirements for registration of antagonistic bacteria u/s 9(3B) and 9(3) of the Insecticide Act, 1968
4.1	Indian standards – antagonistic bacteria – specifications
5	Guidelines/data requirements for registration of entomotoxic bacteria technical and formulation u/s 9(3B) and 9(3) of the Insecticide Act, 1968
5.1	Indian standards – entomotoxic bacteria – specifications
6	Guidelines for minimum infrastructural facilities to be created by the manufacturers of microbial biopesticides (antagonistic fungi, entomopathogenic fungi, antagonistic bacteria, and entomotoxic bacteria)
7	Guidelines for minimum infrastructural facilities to be created by the manufacturers for baculoviruses (NPV, GV)
8	Guidelines for minimum infrastructural facilities to be created by the manufacturers of botanical biopesticides (*Pyrethrum*, azadirachtin, *Cymbopogon*, etc.)

Source: http://www.cibrc.nic.in/guidelines.htm

price. This in turn has created a market for and also encouraged the private commercial production of microbial pesticides (Rabindra and Grzywacz 2010).

10.2.2.1 Registration and the Regulatory Guidelines

10.2.2.1.1 The Central Insecticide Board (CIB)

According to the guidelines of Insecticide Act of 1968, any microbial strain developed or sold for pest and disease control should be registered with the CIB of the Ministry of Agriculture. Inclusion of biopesticides in the Insecticide Act of 1968 has mandated this registration process. Manufacturers of biopesticides can register their products temporarily or regularly. This system reduces commercial barriers to product development. The data requirement for temporary registration is less stringent than for regular registration (Kulshrestha 2004).

Manufacturers have the option to register their newly developed products under either 9(3B) (provisional registration) or 9(3) (regular registration) section of the Insecticide Act of 1968 (Table 10.2). A dossier containing information about the

biopesticide specifications (chemical composition, source, etc.), bioefficacy, toxicology data, packaging, and labeling must be submitted by the manufacturers while applying for registration of the potential biopesticide.

On the basis of approvals in various meetings of RC, the CIB has framed its own guidelines for registration (as of Oct. 05, 2011) for biopesticides. It has also designed minimum infrastructure guidelines expected from biopesticide manufacturers to issue the manufacturing licenses by state licensing authorities. CIBs confirmed quality standards and guidelines must be followed while developing a novel biopesticide product. Special attention should be given to the quality control parameters like moisture content, shelf life, potency of product in terms of LC_{50}, toxicity, and secondary nonpathogenic microbial load. Standard protocols and laboratory tests for assessing these quality parameters have been prescribed by the CIB (Rabindra 2005).

Till date, there are approximately 500 biopesticides available in the Indian market duly registered under the CIB, but quality control measures still remain as the major constrain to their diffusion in agricultural fields. National laboratories and SAUs are conducting extensive research on biopesticides to develop new potential microbial formulations. Their study and experimental results have clearly demonstrated the bioefficacy of biopesticides for pest and disease management. Central and state governments have developed various schemes to promote and encourage the use of biopesticides as replacement of chemical pesticides. This has led to the increase in demands for biopesticides both at state and national levels. However, with increase in demand, there also has been increase in the inclusion of spurious biopesticide products in the market, which prevented the Pesticide Management Bill (PMB) 2008 to pass. The objectives of PMB include:

- To monitor and regulate the manufacture of pesticides
- To assist in sale of pesticide by granting licenses for registration, manufacturing, and selling of pesticides
- Accessing the efficacy and safety of pesticides by pesticide testing laboratories

Few major constraints in implementation of the regulatory system are:

(a) There is still no ordered mechanism or procedure for the reverification of the issued registration certificates for biopesticides.
(b) Manufacturing licenses for biopesticides are issued on paper, but their existence in actual business market remains in ambiguity, and till date there is an absence of proper mechanism to track them.
(c) Various case studies on market scenario confirm the quality concerns, especially from amateur producers who find it difficult to align their products as per the standards set by regulatory bodies.

10.2.3 Commercialization Challenges of Biopesticides in India

There are various challenges to commercialization of biopesticides in the Indian market, and the responsibility to address these challenges lies with the private sector but also with other important arms of the society like academia, state and central government agencies, public and private researchers and funding bodies, marketing professionals, etc. In fact, all the sectors involved in disease control and pest management must join hands and address these commercialization issues together. Various factors affecting the commercialization of biopesticides in the Indian agricultural market are as follows.

10.2.3.1 Awareness and Information Gap

The lack of awareness, knowledge, and confidence in farmers is one of the chief reasons behind the lagging of these eco-friendly pest control alternatives. Biopesticide packs carry detailed instructions and precautions for storage and usage. In spite of this, usage methods are often not clear to farmers. Sometimes the farmers lack the essential skills required for adopting the biopesticides in their agricultural farms.

10.2.3.2 Low Reliability and Inconsistent Field Performance

Lack of stability on the effect of biopesticides has been the primary concern for farmers. Since most of the microorganisms are living, several factors like temperatures, moisture, pH, exposure to ultraviolet radiation, and soil factors adversely affect their efficacy (Arora et al. 2010). Furthermore, the production of biopesticides is prone to contamination, which results in low count of active microorganisms, thus reducing the efficacy of microorganisms and leading to inconsistent field performance (Evans et al. 1993).

10.2.3.3 Poor Quality and Shelf Life

Contamination and low cell count are the main concerns of the farmers and distributers (Alam 2000). It is difficult to maintain the sterile conditions for a longer period. Contamination results in death of active microorganisms, and due to low microbial count, their performance is poorly deprived and uneven. This further leads to low shelf life and inconsistent performance of product in field conditions.

10.2.3.4 Huge Investment and Lack of Profit

Biopesticides are high-technology products which are developed and maintained under completely sterile conditions. A huge capital investment has to be made not only in the initial development stages but also during its packaging, storage, and distribution. Screening of suitable strains and R&D issues add to the budget. Large-scale screening of strains with biological activity is still required (Bashan 1998). In general, firms with larger production facilities are expected to invest more on networks to understand and access the market. Besides, there are high risk and less profit associated with it. All these factors make biopesticide production a costly

business, and companies will only develop these products if there is a long-term profit in doing so.

10.2.3.5 Health and Ecological Risks

Biopesticides may pose some adverse health effects if these are not used according to the guidelines mentioned on the labels of the commercial product. Biopesticides containing *Bacillus thuringiensis* as active ingredient are not reported to show any major adverse effects on human health, but in some cases, occupational exposure has confirmed health risks (Doekes et al. 2004). Studies on fungal biopesticides report that spore of entomopathogenic fungi such as *Trichoderma*, *M. anisopliae*, and *B. bassiana* may cause allergies in immune-compromised patients to farmers (Iida et al. 1994; Darbro and Thomas 2009; Keswani et al. 2014).

10.3 The Indian Market for Biofertilizers: Current Status and Regulatory and Commercialization Challenges

10.3.1 Current Scenario

Biofertilizers are biologically active products or microbial inoculants containing one or more beneficial bacteria, algae, or fungi, with the ability to conserve and mobilize crop nutrients in the soil (Mazid et al. 2011). The microorganisms, which are used as biofertilizers, belong to families of bacteria, blue-green algae, and fungi. These microorganisms are cultured in laboratories and packed into a suitable carrier, after which they are either used for soil application or treatment of seeds. Ionizing radiations are used to sterilize the carriers of the rhizobia and other biofertilizers in storage for a long period (Tittabutr et al. 2012).

Most biofertilizers are categorized into two major groups: nitrogen fixing and phosphate solubilizing. Nitrogen-fixing biofertilizers fix atmospheric nitrogen into simple compounds which can be readily and safely taken up by the plants. Examples of nitrogen-fixing bacteria include *Rhizobium*, blue-green algae (BGA), *Azotobacter*, *Azolla*, and *Azospirillum*. Phosphate-solubilizing bacteria (PSB) secrete organic acids which solubilize the organic and inorganic phosphates and thus enhance the uptake of phosphorus by plants. PSB are particularly valuable as they are not crop specific and can benefit all crops (Table 10.3).

Biofertilizer production is demand driven in nature. Thus, generation of demand among farmers becomes the most crucial step toward promotion of biofertilizers. In India, the promotion and commercialization of biofertilizers began with the launch of National Project on Development and Use of Biofertilizers (NPDB) by the Ministry of Agriculture under the ninth five-year plan. However, the market is still nascent and there is a lot of scope in the future for setting up of biofertilizer-producing units. By now, over 100 biofertilizer units are operational across India, and their capacity is growing exponentially. Figure 10.1 provides a graphical representation of percentage distribution of biofertilizers in different regions of India.

Table 10.3 List of commonly produced biofertilizers in India

Biofertilizer	Trade name	Target crop	Observed benefits
Rhizobium	Jai Vjai Bio-gold	Legumes like pulses, groundnut, soybean	10–35 % yield increase, 50–200 kg N/ha
Azotobacter	JIBANU-SARA	Soil treatment for nonlegume crops including dry land crops (wheat, rice, vegetables)	10–15 % yield increase – adds 20–25 kg N/ha
Azospirillum	GROTOP	Nonlegumes like maize, barley, oats, sorghum, millet, sugarcane, rice, etc.	10–20 % yield increase
Blue-green algae	Skipper Khad	Rice/wet lands	20–30 kg N/ha, *Azolla* can give biomass up to 40–50 tonnes and fix 30–100 kg N/ha
Phosphate-solubilizing bacteria (PSB)	Phospho Shakti	Soil application for all crops	5–30 % yield increase

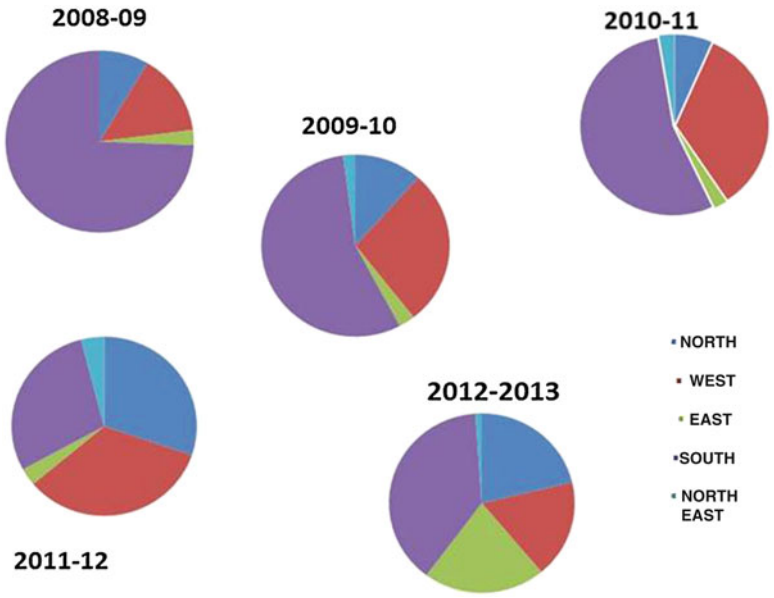

Fig. 10.1 Pie chart showing percentage distribution of biofertilizers in different regions of India

10.3.2 Regulatory Framework and Regulation Constraints for Biofertilizers in India

Indian legal framework related to biofertilizers is quite robust. The Indian Ministry of Agriculture issued an order in 2006 which included biofertilizers under the Essential Commodities Act of 1955.

10.3.2.1 Quality Control and Standards of Biofertilizers

The quality check of any new biofertilizer is a must before it diffuses in agricultural field and gains acceptance by the farmers. To assure proper quality of a biofertilizer product, the legislation has set some guidelines which must be strictly followed and reflected in the labeling requirements. In India, the Ministry of Agriculture and Farmers Welfare has prescribed production and marketing standards with respect to the various types of microorganisms forming the biofertilizer. There are seven major quality parameters which are discussed in Tables 10.4 and 10.5. Groups of

Table 10.4 Specification of *Rhizobium* biofertilizers

(i)	Base	Carrier based* in form of moist/dry powder or granules or liquid based
(ii)	Viable cell count	CFU minimum 5×10^7 cell/g of powder, granules or carrier material or 1×10^8 cell/ml of liquid
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5–7.5
(v)	Particle size in case of carrier-based material	All material shall pass through 0.15–0.212 mm IS sieve
(vi)	Moisture percent by weight, maximum in case of carrier based	30–40 %
(vii)	Efficiency character	Should show effective nodulation on all the species listed on the packet

Source: Biofertilizers and organic fertilizers in fertilizer (control) order, 1985 national
**Type of carrier*: the carrier materials such as peat, lignite, peat soil, humus, wood charcoal, or similar material favoring the growth of organism

Table 10.5 Specification of mycorrhizal biofertilizers

(i)	Form/base	Fine powder/tablets/granules/root biomass mixed with growing substrate
(ii)	Particle size for carrier-based powder formulations	90 % should pass through 250 μm IS sieve (60 BSS)
(iii)	Moisture content percent maximum	8–12
(iv)	pH	6.0–7.5
(v)	Total viable propagules/g of product, minimum	100/g of finished product
(vi)	Infectivity potential	80 infection points in test roots/g of mycorrhizal inoculum used

microorganisms considered include *Rhizobium*, mycorrhizal fungi, phosphate-solubilizing bacteria (PSB), *Azospirillum*, and *Azotobacter*. Tables 10.4 and 10.5 give a detailed specification of *Rhizobium* and mycorrhizal biofertilizers, respectively, as per the prescribed guidelines.

10.3.3 Commercialization Challenges of Biofertilizers in India

10.3.3.1 Marketing of Biofertilizers

The marketing of biofertilizers in India depends upon many factors like perceptions of farmers, government intervention, and difficulties in using the biofertilizer technology. Ghayur (2000) conducted a field study in two districts, Karnal and Bhiwani (Haryana), to find out various reasons behind low acceptance of biofertilizers in agricultural field of India. The study reported that in spite of the government's efforts to promote biofertilizers, the farmers are still reluctant in accepting it due to problems of unavailability and poor quality. The state agricultural departments and shopkeepers are unwilling to stock and sell biofertilizers due to lack of its stability and inconsistent field performances. In addition, the low demand for biofertilizers has prevented investment in advanced production and storage facilities, which are required for improving the quality.

The study concludes that the present policy of providing grants and low-interest loans to biofertilizer producers should be abolished, as it has led to setting up of a large number of inefficient plants, which cannot produce good quality biofertilizers. The policy of marketing biofertilizers at very low prices should also be stopped, as these prices are too low to attract modern investment in modern manufacturing units. In addition to this, farmers feel that nothing so cheap can provide much nutrition to the plants.

10.3.3.2 Certification and Quality Control

The utility of biofertilizers has been validated by reputed agencies in India (such as NBDC and ICRISAT) and abroad. For instance, field trials by NBDC have showed that application of *Azotobacter* results in 3–25 % increase in yield in cotton and 2–20 % in wheat, in Haryana. But biofertilizer manufacturers find it difficult to consistently replicate results of biofertilizer usage. This is because agroclimatic conditions and soils vary in different parts of the country. Many strains do not survive in very hot temperatures. Biofertilizers are also prone to contamination if carriers (such as powder/liquid) are not sterilized. If farmers do not get consistent quality of biofertilizers, it impacts the yield. This further reduces their trust and consequently the purchase of biofertilizers. In addition, though biofertilizers have to conform to BIS standards, there is no proper certification process in place.

10.3.3.3 Empirical Findings of Case Studies

Despite positive signs for green agriculture in India, the growth of biofertilizer industry and consumption rate has not been impressive. To address the issue of low diffusion of biofertilizers in agricultural field, two organizations, namely, Bharatiya

Agro Industries Foundation (BAIF) and Kumar Krishi Mitra Bio Products Pvt. Ltd. (KKM), carried out an extensive field exercise. The media used are pamphlets, brochures, audiovisual material, advertisements, demonstrations and setting up of trial/demo farms, dealer workshops, and discussion forums. They carried out interviews with farmers, dealers, extension workers, and officers. The empirical findings and observations from these exercises from multiple stakeholders' perspectives are listed as follows.

10.3.3.3.1 Producers'/Distributors'/Traders' Perspective

According to this group, the main constraint to the access of biofertilizers by farmers is the lack of appropriate infrastructure for its storage and distribution. Lack of proper quality control mechanisms has resulted in inclusion of poor quality biofertilizers in market. This contributes to the poor performance of biofertilizers in agricultural field and increases the ambiguity among the farmers.

10.3.3.3.2 Farmer's (Users') Perspective

Lack of awareness and essential skills required for adopting the biofertilizers in their agricultural farms has been the major concern for the farmers. Because of limited shelf life and inconsistent field performance, biofertilizers are perceived as less yielding. Besides some climatic regions and soil conditions are not suitable for specific strains in organic production.

10.3.3.3.3 Government's (Promoters') Perspective

Since the inception of green revolution, there has been increased use of chemical fertilizers to increase food production in the country and thus make it self-sufficient. In the process of achieving this development goal, all the agricultural departments, research institutions, and extension services have for long been oriented toward chemical input agriculture. Now to bring about a sudden paradigm shift to organic agriculture will definitely take some time. Moreover, changing the cropping and cultivation patterns is slow and time-consuming in nature. Subsidies on chemical fertilizers and pesticide obturate the growth of organic agriculture.

10.4 Conclusion

After numerous group discussions with different stakeholders of organic inputs (farmers, traders, government officials, agricultural scientists, extension officers, and NGOs) and taking into consideration their varied perspectives, few general recommendations or guidelines to enhance promotion and acceptance of green agriculture in India are suggested. In addition, to facilitate the diffusion of biopesticides and biofertilizers in Indian agricultural scenario, various national strategies have to be formulated.

The government should intervene and take necessary steps for promoting and funding research for developing novel or improved strains suitable for different soil and environment conditions, especially for cereal crops. To develop high-quality

products with improved shelf and field life, emphasis should be laid on developing novel formulations and carrier materials. We need to develop safety indices for biopesticide and biofertilizer formulations including acceptable levels of inorganic contaminants. There should be a strict and transparent monitoring of quality of biopesticides and biofertilizers at various stages of production, marketing, procurement, and applications to avoid diffusion of spurious products in the market. For achieving this, proper effort should be taken in developing more testing laboratories with adequate infrastructure and manpower. For bioefficacy testing, referral labs with good infrastructural facilities may be notified by the CIB. Besides, the CIB should form a technical expert committee comprising of scientists to oversee the infrastructural facilities of biopesticide and biofertilizer production units before granting registration under section 9(3B) or 9(3). The existing production units should be subjected to accreditation. Also the development of farmer-friendly technology should be the prime focus of all the research.

To reduce farmers' risk and increase acceptance of biopesticides and biofertilizers by them, some insurance or buyback of product scheme should be initiated. The cost of such schemes can be jointly shared by the government and the manufacturing firm. Public-private collaboration in research, production, and commercialization should be encouraged. The industry must promote standards for biopesticides and biofertilizers to translate their value in agriculture, forestry, and other target markets. A strong academic-industry alliance is necessary for scaling up the commercialization of biopesticides and biofertilizers. A road map may be developed for putting this agenda into implementation.

Indigenous knowledge of bio-inputs should be validated and documented and its integration with scientific knowledge should be undertaken. There should be development of data banks freely accessible for reference and use. Therefore, it is imperative to bring out manuals for supporting the development of high-quality biopesticides and biofertilizers. Harmonization of international regulations is required. The fact that the use of biopesticides and biofertilizers is a knowledge-intensive input is highlighted. The issues of data protection and IPR must be addressed. Registration requirement for biopesticides and biofertilizers could be relaxed and rationalized. Since some of these agents have the ability to increase plant growth, they should be considered as plant growth-promoting agents for relaxing registration requirements like toxicological data. There is an urgent need to develop bar coding of microbes used in commercial production of biopesticides and biofertilizers. Microbial consortia can be developed for better results. Accordingly, the CIB may amend the existing rules for biopesticide and biofertilizer registration. Selection of proper strain/species of biocontrol agents is the key factor for overall success. More focus may be given to develop low-cost technologies for mass production of biopesticides and biofertilizers. Attention should be given to develop postharvest disease management practices. A concerted effort of research institutes, universities, nongovernment organizations (NGO), and government organizations is required to elevate the stature of biopesticides and biofertilizers which in turn will facilitate their diffusion in the Indian market.

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Regulatory Requirements and Registration of Biopesticides in the Philippines

11

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Abstract

Traditional agriculture, in a perpetual effort to maximize productivity, have always relied on synthetic pesticides to control pest infestations. However, usage of these synthetic chemicals has an inadvertent adverse effect on the environment. Thus, they are not sustainable and there is a need to slowly decrease its usage in favor of pesticidal products that are more environment-friendly in nature. An alternative to traditional pesticides is biological pesticides or biopesticides. Biopesticides are biocontrol chemicals derived from natural resources such as plants, animals, minerals, or microorganism such that the usage of which is without the threat of environmental contamination and pollution. Moreover, most biopesticides have been proven to be at par if not better relative to the dominant synthetic pesticides in the market. Thus, biopesticides have been constantly promoted as an alternative to traditional and inorganic pesticides. Despite this, biopesticide usage in the Philippines and the world in general remains relatively diminutive. Inefficiencies in registration of new biopesticide products are in part responsible. Regulation of pesticides and biopesticides in general is governed by the Fertilizer and Pesticide Authority (FPA) agency in the Philippines. The lack of general interest in biopesticides locally has been attributed to insufficient trainings and extensions to farmers, insufficient manufacturing capacity to satiate even the meager demand, lack of biopesticide inoculant resources repository, limited to absence of linkages between local government units and farmers, and cultural tendency – the 50-year-old habit of the massive application of fertilizers and pesticides brought about by the practices in green revolution. Recommendations to promote local biopesticides utilization include partnerships with the private sector to facilitate mass production and commercialization if government or state-owned biopesticides formulation plant is not possible,

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establishment of biopesticides inoculant resources repository, and improvement and maintenance of the quality of trainings and seminars provided to farmers.

Keywords
Biopesticides • Formulation • Fertilizers • Pesticides

11.1 Introduction

Pest-induced stress and infestations on crops has had a tremendous impact on the productivity of crops throughout the history of agriculture. Various outbreaks have led to widespread destruction of food crops ultimately leading to famine and the death of millions of people. This is especially true for underprivileged countries where the economy heavily relies on agriculture. Thus, it is of utmost importance to minimize and manage the damage brought about by such infestation. Pest infestations have been generally managed through the use of traditional inorganic pesticides. The first generation of synthetic pesticides is in the form of highly toxic compounds, such as arsenic and cyanide-based chemicals. Later on, it was deemed highly unsafe leading to the dawn of synthetic organic compounds. The application of these organochlorine compounds with a broad-spectrum effect, however, has a huge negative impact on non-target beneficial organisms. Moreover, these compounds are highly stable and tend to persist in nature leading to the accumulation and magnification in biological systems. The Philippine pesticide industry emerged in the 1950's, coinciding with the popularity in usage of the said compounds such as DDT, 2,4-D, endrin, and malathion (Elazequi 1989).

Although traditional methods of pest control have made a huge impact in the control of pest infestations, the negative ecological impacts cannot be ignored. An eco-friendly alternative to broad-spectrum traditional chemical pesticides is biopesticides. According to FPA (2016), they can be in the form of biochemical pest control agents including pheromones, kairomones, allomones, and hormones; natural plant regulators such as auxins and enzymes; and microbial pest control agents such as bacteria, fungi, protozoa, and virus-based products. It has the advantage of having no chemical residues and thus has no known adverse effect on the environment (Gupta and Dikshit 2010; Keswani et al. 2014; Bisen et al. 2015). In addition, most biopesticides have a narrow spectrum of effect and are thus not lethal to non-target organisms.

The Philippines is largely an agricultural country where agriculture accounts for 11.3 % of the current GDP according to World Bank (2014). In addition, 24 % of the total export and 46 % of the total employment are from agriculture (Bureau of Agricultural Statistics, 2003). Important agricultural commodities of the Philippines include rice, corn, mangoes, pineapples, and coconut largely because they either are a staple food crop or they comprise a large proportion of the agricultural export (Bureau of Agricultural Statistics, 2004). Pesticide application has been a major factor in the increase of productivity for these crops. It is estimated that in 1990, pesticide industry in the Philippines is valued at around 100 million USD - 40 % of

which was used on rice whereas 20 % was allocated for vegetables (Gaston 1994). Small-scale farmers which comprise the bulk of the agricultural industry in the Philippines are heavily reliant on old and broad-spectrum effect pesticides. This is understandable because these synthetic formulations are inexpensive, easily applied and as well as effective. Biopesticides remain largely overlooked despite considerable effort put into research and extension by various local institutions. In spite of its lukewarm success, the Philippine biopesticide industry has a wide array of products commercially and locally available.

11.2 Locally Available Biopesticides in the Philippines

11.2.1 Biofertilizers as Biocontrol Agent

Biofertilizers are not only limited in function as plant growth enhancers but also act as a preventative measure against pathogen infection. Some of these products are Vesicular Arbuscular Mycorrhizal Root Inoculant (VAMRI) (Fig. 11.1). It is composed of chopped dried corn roots infected with *Glomus mosseae* or *Glomus fasciculatum*, an arbuscular mycorrhizal fungus. It can serve both as a biocontrol agent and a biofertilizer. The biological agent allows for a better absorption of nutrients especially non-mobile elements such as phosphorus and zinc. VAMRI greatly reduces disease incidence caused by *Fusarium oxysporum*, *Pythium* spp., *Phytophthora infestans*, and *Ralstonia solanacearum*. It also induces resistance against burrowing nematodes such as *Radopholus similis* in banana seedling plants. However, the degree of effectiveness of VAMRI varies depending on the plant host, variety, cultivar, VAM inoculant, density of application, and soil fertility. It has been found to be effective inoculants for various crop species such as *Capsicum* spp. (pepper), *Solanum melongena* (eggplant), *Lycopersicon esculentum* (tomato), *Carica* spp. (papaya), *Musa* spp. (banana), *Ananas comosus* (pineapple), *Citrullus lanatus* (watermelon), *Allium cepa* (onion), *Zea mays* (corn), *Saccharum officinarum*

Fig. 11.1 Commercial packaging of VAMRI as marketed in the Philippines (Javier and Brown 2007)

Fig. 11.2 Commercial packaging of Brown Magic

(sugarcane), *Arachis hypogaea* (peanut) and some fruit crops and ornamental plants. Application of VAMRI can potentially replace 50–100 % of the fertilizer requirement depending on the aforementioned factors. Currently, VAMRI is widely used by onion farmers in the northern part of the Philippines. Trainings and extensions have been conducted regarding its use across the Philippines, although it is still insufficient owing to financial constraints (Brown et al. 2013, 2006, 2002, 1998).

Another BIOTECH-UPLB product primarily used as a plant growth enhancer is the Brown Magic (Fig. 11.2). The product is also a mycorrhizal-based inoculant used for orchids. It acts as a biofertilizer as well as biocontrol agent against soil-borne diseases of orchids. The fungal inoculant is composed of sclerotium or fruiting bodies of fungi and mycelia collected and isolated from orchid roots. Application of Brown Magic increases orchids' tolerance and resistance against pathogens and diseases. As a biofertilizer, it induces early flowering in orchids, production of more suckers and longer spikes.

Mykovam on the other hand is a soil-based biofertilizer that can also act as a biocontrol agent. It contains spores, infected root propagules, and VAM fungi. The mode of action in growth promotion is similar to VAMRI – it increases the effectiveness of nutrient absorption. A positive side effect of inoculation is the increased tolerance to pathogens, environmental stresses such as water deficit and heavy metal exposure. The product is economical and relatively easy to use. It only needs to be applied once, and it has the capacity to replace 60–85 % of the commercial fertilizers used to plant growth. The product can be applied to a wide array of plants such as vegetables, fruit crops, trees, and ornamentals with the exception of orchids.

Biogroe, on the other hand, consists of plant growth promoting rhizobacteria (PGPR) that positively influence root growth as well as solubilize nutrients. The presence of PGPR in the roots also influences the plants' capacity to defend itself against pathogens through out competition and production of metabolites inhibitory to the growth of pathogens. It can be used on crops such as rice, sugarcane, vegetables, and some ornamentals.

Biocon is marketed as both a biofertilizer and biofungicide. The biological agents are three *Trichoderma* spp. including *T. parceramosum*, *T. pseudokoningii*, and *T. harzianum*, acquired through strain improvement *via* UV irradiation. *Trichoderma* spp. are a well-known inhibitor of a wide range of fungal pathogens. The product is applied through seed coating prior to direct seeding or as a soil inoculant. It enhances absorption of mineral nutrients and can replace the fertilizer requirement of plants by up to 50 %. The use of Biocon requires minimal application of fertilizer since the effectiveness of the product decreases in the presence of large amount of chemicals. One-time application of Biocon is sufficient to decrease damping off disease caused by *Pythium* spp., *Sclerotium rolfsii*, and *Rhizoctonia solani.* Moreover, the presence of these beneficial fungi on soil at high population density correlates with increased germination and survival of *Brassica chinensis.* Biocon-treated seeds have a higher incidence of seedling survival and germination in the case of tomatoes and celery grown in high-altitude areas in the Philippines compared to those treated with mancozeb, a commercially available fungicide. Mass production and commercialization of Biocon was done through partnership with TriGeo Technologies, Pvt. Ltd. (Cuevas et al. 2005).

11.2.2 Biopesticides

Biopesticides are subdivided into three major classifications namely: microbial, plant, and biochemical pesticides.

11.2.2.1 Microbial Biopesticides

Microbial biopesticides that are currently available in the Philippines include those possessing entomopathogenic fungi, *Beauveria bassiana* (Bals.) *Aspergillus versicolor* (Vuill.), and *Metarhizium anisopliae* (Metsch.). The mode of action can either be through outcompeting the pathogen or through the production of toxic compounds lethal to the pathogen. *B. bassiana* and *M. anisopliae* Sorok cause the white and green muscardine diseases in insects. The fungal species grows in the cuticle of the insect penetrating its inner body where toxins are released leading to the death of the insect. The carcass of the insect is further degraded and covered with white downy mold where newly infective spores are propagated and released into the environment. The entomopathogenic fungi are effective against a wide array of insect pests, and their biocontrol potential have been evaluated against important pests in the Philippines such as the Asian corn borer (*Ostrinia furnacalis*) and coconut hispine beetle (*Brontispa longissima* Gestro) (Javier and Brown 2007).

Nuclear polyhedrosis virus (NPV) on the other hand is used to eradicate worms and larvae particularly those with basic gut pH. It infects the target through the ingestion of contaminated plant parts. The capsid of NPV degrades at alkaline pH releasing the viral genome and proteins allowing for infection. It targets organisms with basic gut pH; thus, it is safe for humans and organisms with acidic gut pH which renders the activation of the virus, impossible. The infected larvae excrete droppings that are contaminated with virus, thus facilitating further dispersal and

Fig. 11.3 Commercial packaging of Bactrolep

increasing range and effectiveness, directly proportional to the severity of the infestation (Lavinaa et al. 2001; Adams and McClintock 1991).

The utilization of bacterial toxins has also been explored for the biocontrol of Lepidopteran insect pests in the form of Bt toxin. The mechanism is similar to NPV, such that the toxin is only activated at alkaline pH. The toxin incapacitates the target by affecting the digestive system leading to deprivation and ultimately death. Bactrolep is a Bt-based formulation that is especially effective against Asian corn borer (*Ostrinia furnacalis*) and diamond back moth (Fig. 11.3) (Padua et al. 1990, 1984, 1980). Asian corn borer infestation accounts for 4–30 % of yield loss during dry season which is magnified during wet seasons (Gonzales 2000).

11.2.2.2 Plant Biopesticides

Plant-derived pesticides are mainly defined as plants wherein the resistance itself was incorporated to the plant through genetic modification such is the case in Bt corn. In this case, the gene for the Bt toxin is in the corn genome and expressed constitutively throughout the plant, though some may use promoters restricting the expression in specific tissues. It is found that the use of Bt corn translates to an increase in yield of 34–37 % in Camarines Sur and Cotabato (Yorobe and Quicoy 2006). It has also been shown to have a higher quality of grains owing to the minimal quantities of mycotoxins in the kernel (Wu 2007). Currently, Bt corn is locally not accepted for human consumption and is only allowed for the production of animal feeds. Several plant species susceptible to Lepidopteran infestation have been the subject of Bt gene incorporation. These include soybean, eggplant, and cotton.

Currently, the development and usage of genetically modified plants are under stringent regulations in the Philippines.

Another plant-derived pesticide that was developed in the Institute of Plant Breeding in UPLB is the transgenic papaya resistant to papaya ringspot virus (PRSV). The virus causes papaya mosaic disease which affects not just papayas (*Carica* spp.) but cucurbits in general. Infected plants develop mosaic and chlorosis in the leaves. Advanced infection exhibits young leaf distortion leading to a shoestring appearance, which could be mistaken as mite infestation. Infection at an early stage of the plant shows symptoms of stunted growth. Fruits from the infected plants, on the other hand, manifest ringspots. The development of the genetically modified papaya resistant to PRSV was through the use of coat protein (cp) gene technology conducted through particle bombardment. The technology made use of a fragment of CP gene derived from Philippine PRSV strain (Magdalita et al. 2004).

11.2.2.3 Biochemical Biopesticides

Biochemical pesticides on the other hand refer to substances that are naturally occurring in nature having pesticidal qualities. The mechanism does not involve toxicity, but rather it affects the target organism's behavior leading to growth or reproduction disruption. Some substances belonging to this category include hormones which affect the target through the spatial separation of males and females during mating season and synthetic compounds that are used to lure the target insect away from food crops affecting their feeding and foraging abilities. Such is the case in *Sternochetus frigidus* (Fab.) or mango pulp weevil which causes widespread mango floral devastation during blooming periods (De Jesus and Gabo 2000). Mango pulp weevils feed on mango floral parts, nectar, and pollen. The weevils are attracted to the volatile chemicals emitted by *Mangifera indica* (mango) at full floral bloom. It has been subjected to gas chromatography – mass spectrometry (GC-MS) in an attempt to elucidate the putative chemicals serving as an attractant to the weevils.

Allelopathy, or inhibition of growth of organisms by another in its vicinity due to the release of chemical byproducts indirectly affecting growth or reproduction of an adjacent species has also been thoroughly studied and used in the Philippines. The inherent properties of such plants serve as a deterrent to some insects. For instance, *Tagetes erecta* emits a metabolite that is lethal against insect larvae and as well as some nematodes. Morallo-Rejesus (1984) led a study on this topic and in that she identified 34 species of plants with potential insecticidal properties, 27 of which were found to be toxic to more than one insect species. Some notable plant species identified were *Tinospora rumphii* Boerl., identified to be an effective deterrent against green and brown plant hopper (Leonardo 1983) and *Piper nigrum* against diamondback moth larvae, adult housefly, cotton stainer, corn weevil, and black armyworm (Javier and Morallo-Rejesus 1986).

11.3 Philippine Biopesticide/Pesticide Registration Requirements

Section IX of the Presidential Decree 1144 promulgated in May 1977 marks the establishment of the Fertilizer and Pesticide Authority (FPA) responsible for the regulation of the quality of pesticides/biopesticides, such that the commercially released pesticide has minimal health and environmental hazard and ensures that its price is set reasonably. Consequently, all pesticides and biopesticides as well as their handlers have to be registered under FPA, prior to product release and commercialization. Under FPA, the Pesticide Technical Advisory Committee is responsible for governing the registration, setting of the requirements, issuing labeling guidelines, approving or disapproving of license application, handing out certification on approved pest control operators, regulating imports, and limiting the availability of toxic agricultural products. The detailed registration process under FPA is shown in Fig. 11.4 (FPA 2016). The registrant is required to submit a technical grade sample of the formulated product along with an application, data package, and samples of the technical material and analytical standards to FPA. The data package includes prior information on the identity of the registrant, product itself, manufacturing process, product composition, physical and chemical characteristics, bioefficacy data, toxicological data which includes information on long- and short-term toxicity studies, human safety data, environmental hazard, and transport and residues in food. The application is then reviewed by experts from different institutions and universities where the results and recommendations are submitted to the Technical Advisory Committee. The committee then forwards the recommendations to FPA which then approves or disapproves the registration. If the application is approved, further instructions on the labeling pictorially depicting hazards will be required in compliance to World Health Organization's recommendation. Each registration is subject for renewal every 3 years.

Unfortunately, regulation of pesticidal products under the FPA has not been rigidly enforced. As a result, banned products still lurk the market, and their residues are still detected on biological systems. This is in part due to the financial constraints leading to the inability to enforce its mandate. An added effect of this constraint would be product analyses inefficiencies (Lu et al. 2010).

Biopesticides are biocontrol chemicals, the active ingredient of which has been derived from natural resources such as plants, animals, or microorganisms. Owing to the nature of its source, the mode of action of biopesticides is considerably different than that of traditional inorganic and synthetic chemicals. Data requirements for the registration of such needs to be different as well. Some types of biopesticides that is generally required to be registered are botanical pesticides, microbial pesticides, and biochemical and macrobials such as biocontrol insects. Southeast Asian nations in general have a few biopesticides that are registered in the form of neem and rotenone.

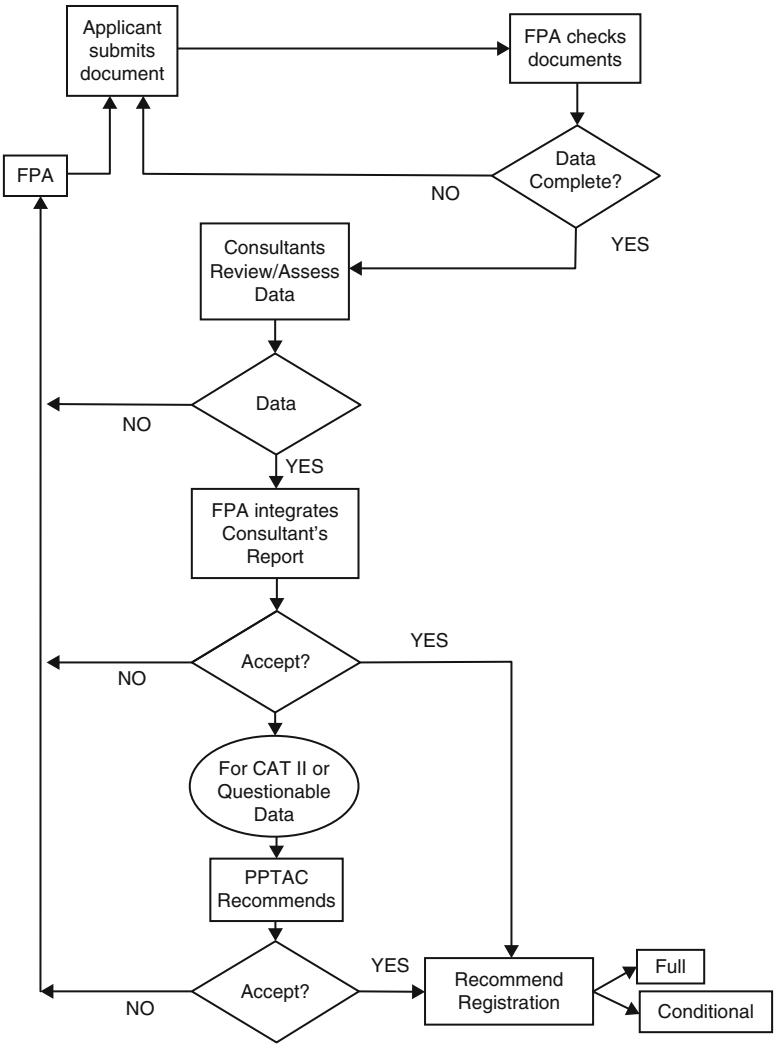

Fig. 11.4 Schematic diagram of the pesticide registration process under the FPA (FPA 2016)

There has been an effort to harmonize pesticide and biopesticide registration requirements and process in Asian countries in line with the provisions of FAO Code of Conduct in Pesticides (FAO 2012). A guideline was developed governing the registration of phytochemicals, pheromone, and microbial biopesticides. It contains pertinent information on the suggested requirements for botanical, microbial, parasitoid, and pheromone-based biopesticides.

11.4 Constraints and Recommendations on the Use of Biopesticides

The root cause of the meager consumer demand and usage of biopesticides in the Philippines lies in part with the regulatory policies regulating the registration of pesticide and biopesticide products, administrative policies subsequent to the development of technologies, and absence of well-defined linkages between the local government units and farmers.

The regulatory policies associated with the regulation of pesticides and biopesticides in the Philippines present stringency in biopesticide registration even though it is a well-known fact that it, costs less, is eco-friendly and sustainable in nature. This is because biopesticide registration process is almost similar to the registration process of traditional pesticides. Consequently, the registration of new biopesticides would cost a sizable amount of money. It is therefore recommended that the cost of biopesticide registration be atleast lower especially if the registrant is a government research institution or university. This is reflected on a very limited number of biopesticides that have been registered under FPA as compared to the vast array of synthetic pesticides registered though it is due to a very limited number of researchers developing and testing new biopesticides. This presents the area that require urgent attention. Moreover, there is limited access to researchers on biological resources for biopesticides development. Thus, it is recommended that a regional bank for starter inoculants concerning biopesticides be established. It would function as a repository of biological materials found having the potential for use in biopesticide development.

In the Philippines, the support of the development of a product or technology ends with the product development. At this point it is up to the researcher to seek grants in order to conduct further research, extensions, and seminars to increase the public awareness regarding the availability, advantages, and disadvantages in the usage of the product. Failure to do so means that limited investments on the product will be made. Bridging this gap between the researchers and farmers, most of the time, is the burden of the researchers. The tendency therefore is that most products tend to be manufactured in the research institution itself, which in most cases lack the capacity for mass production. Due to this reason, biopesticides in general are not readily available in the market unlike synthetic pesticides. Moreover, added to the burden of manufacturing is the cost of informing, conducting seminars, and advertising which serves to increase the awareness of the public on the product. An alternative to commercialization could be through the establishment of linkages with the local government units and nongovernment offices. The linkage will aggressively extend the technology to farmers through integrated pest management programs in the Philippines.

Biopesticides with its narrow spectrum of effect tend to make the application procedures more complex, such that the farmers without the proper trainings will naturally be daunted and intimidated by the task of using and applying biopesticides. This is in stark contrast to traditional pesticides wherein application tends to be the same way – spray as much as you can in an area as wide as you can. In

addition, most farmers have the unfounded notion that biopesticides are less effective than traditional pesticides (Rola 1990). More importantly, farmers are used to the practice of application of traditional pesticides. The green revolution half a century ago has led to the wide usage of high-yielding varieties exceedingly dependent on the massive usage of inorganic fertilizers and pesticides to maintain high yields. Such is the case in *Masagana 99* during the 1970s wherein high-yielding varieties of rice have been widely promoted nationwide despite limited information on its pest susceptibility. Moreover, cultural tendencies of the locals are inclined toward religiosity and superstition. The dominant religion therefore has a high degree of influence and the power to impose its stand on various issues, such as its opposition to GM crops. Biopesticides in the form of GM crops therefore have a very limited acceptance in the Philippines. Recent court ruling on Bt eggplant has prohibited further field testing trials in the country citing the reason as lack of scientific consensus on the risk involved. GM crops have been therefore frequently presented as a technology that poses a high degree of risk. Consequently, the locals in general naturally have an unfounded aversion and reluctance to use the technology.

Commercialization, after product development, is another facet that needs to be critically addressed. Of the multitude of biopesticides and biofertilizers developed in the Philippines, only a few have gained private investment for manufacturing and commercialization. The reality is more grim for biopesticides as compared to that for biofertilizers as it faces a far more competitive market with a myriad of fast-acting and broad-ranged pesticides. Traditional synthetic pesticides in the Philippines are manufactured en masse by multinational companies with streamlined production and commercialization process. Thus, their products are inexpensive, although not necessarily affordable to most local farmers, widely available, and widely known. Investments should be made by the government and partnerships with the private sector must be promoted. In this approach, private-owned manufacturing would have the prerogative to set prices with the intent of competitively maximizing profit. Thus, it is recommended that a research institution be granted the benefit to create an establishment which would serve as the factory or mixing plants for the biopesticides. This arrangement will allow for the maximum benefit for the research institution and the farmers.

Trainings and extensions must be conducted in various parts of the country so that pesticide users are well informed of the advantage of using biopesticide and biofertilizers. Consequently, relevant and comprehensive training must be conducted for the trainers to ensure the quality of the trainings. This endeavor is to guarantee that farmers who will attend the training program will have the capacity to plan and decide their strategy for pest and disease management. The farmers must be aware of the proper application and amount of pesticides to use whether using biopesticides or traditional synthetic pesticides.

Although research on biopesticides started three decades ago, it was only in 2011 that its promotion and wide adoption were implemented through the Republic Act 10068 or Organic Agriculture Act. The formation of the committee to develop guidelines and protocols for the certification and registration is still ongoing in the form of Bureau of Agriculture and Food Standards.

11.5 Conclusion

An alternative to traditional inorganic synthetic pesticides is biopesticides which are derived from natural resources in the form of microorganism, plants, animals, or minerals. In contrast to traditional pesticides, biopesticides are generally safe for use and does not pose adverse environmental risk. Thus, research institutions have poured a great deal of resources in the development of biopesticides. Moreover, they have been constantly promoted for wide usage. Despite this, biopesticide usage remains relatively stagnant, the reason for which has been thoroughly discussed in the previous chapter. In addition, an agency that governs registration and regulation for biopesticides remains ill funded to the point of incapacity to enforce its mandate. In fact, it was only recently that a comprehensive standards for the regulation of biopesticides were crafted in the Philippines. Efforts to harmonize or to establish analogous registration process have been developed by FAO; however, complete adoption of the suggested regulation has not been fully done.

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Biofertilizer Research, Development, and Application in Vietnam

12

Pham Van Toan

Abstract

Biofertilizers are products containing living microorganisms having the ability to convert nutritionally important elements from unavailable to available form and enhance the plant growth through biological processes. The book chapter summarized the research, development, application, and regulatory requirements of biofertilizer in Vietnam and contains the results of isolation of nitrogen-fixing (*Rhizobium*, *Azotobacter*, *Azospirillum*), phosphate-solubilizing (*Bacillus*), and pathogen-antagonistic (*Bacillus*, *Pseudomonas*) microorganism, the evaluation of their biological activities, the effect on the growth, the yield of crops, and the economical effects to the crop production. Briefly informations about the regulatory requirement of biofertilizer in Vietnam are also included in the paper.

Keywords

Nitrogen fixation • Phosphate solubilization • Pathogen antagonistic • Biofertilizer • Multifunctional biofertilizer

12.1 Introduction

Fertilizers play an important role in agriculture and are accountable for about 40 % increase in crop yield. Vietnam is predominantly an agricultural country with more than 10 million ha of agricultural land, so the demand for fertilizers is high. For the past 10 years, the mineral fertilizer supply has been exponentially increasing. However, the fertilizer use efficiency (FUE) is still low which is estimated at

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Table 12.1 N fixation capability of some rhizobial strains

Rhizobial strains	ARA activity (nmol/plant/h)	Plant dry matter (gm/pot)
MAR 377	1920.4	3.90
TAL 1000	247.8	3.18
TAL 236	3933.4	3.87
NC-92 (IC-7001)	672.7	3.60
IC-7029	1207.9	4.55
5a/70 (IC-7017)	495.6	3.55
98	123.9	3.78
THA 201	5513.0	4.38
TL 6-2	997.8	4.17
TL 3-1	4970.2	4.97
Control (non-inoc)	0.0	2.37

40–45 % for nitrogen, 25–30 % for phosphorus, and 60 % for potassium (Nguyen Van Bo 2014).

Biofertilizers contain beneficial microorganisms, which are applied to seed, plant surfaces, or soil, colonize the rhizosphere (region around the surface of the roots) or the interior of the plant, and promote growth by increasing the supply or availability of primary nutrients to the host plant. Subsequent application of biofertilizer reduces dependence on chemical fertilizers, thereby allowing the farmers to cut the cost of agrochemical input.

Research on N-fixing and P-solubilizing microorganisms in Vietnam started two decades ago. The results of the study showed that N-fixing inoculant can reduce nitrogen application by 30–60 kg N/ha/year and increase the crop yield by 5–25 % (Tables 12.1, 12.2, 12.3, 12.4, 12.5, 12.6, 12.7, 12.8, and 12.9). P-solubilizing inoculants and rock phosphate can replace 30–50 % mineral phosphorus fertilizer without significant change in crop yield. This chapter will discuss the environment biofertilizer research, development, application, and regulatory requirements of biofertilizer in Vietnam based on the research projects (Nguyen Kim Vu 1995; Pham Van Toan 2000, 2005; Luong Huu Thanh and Pham Van Toan 2008).

12.2 The Biofertilizer Research, Development, and Applications in Vietnam

12.2.1 Isolation and Selection of Beneficial Microbes

From governmental budgets and international budgets in the last two decades, many research projects were carried out in Vietnam with more than ten research institutions and universities and more than 100 scientists working on the biofertilizer

Table 12.2 Plant nitrogen content of inoculated groundnut 1686

Rhizobial strains	Plant nitrogen content (%)	(mg/pot)
MAR 377	2.21	86.17
TAL 1000	2.77	87.94
TAL 236	2.35	01.06
NC-92 (IC-7001)	2.63	94.68
IC-7029	2.02	91.93
5a/70 (IC-7017)	2.13	71.36
98	2.41	90.97
THA 201	2.07	89.01
TL 6-2	2.24	93.33
TL 3-1	2.07	102.8
Control	2.18	66.12

Table 12.3 N-fixing activity of rhizobial strains

Rhizobial strains	Plant nitrogen (mg/pot)	Ration of ^{15}N-enriched and total plant nitrogen content (%)	N-fixing activity (%)	(mg/pot)
Control	51.67	0.285	–	18.2
MAR 337	86.17	0.225	21.1	21.7
TAL 1000	87.94	0.215	24.6	24.2
NC-92	94.68	0.212	25.6	24.6
98	90.97	0.208	27.0	24.7
THA 201	89.01	0.206	27.7	24.8

Table 12.4 N fixation capability of some *Azotobacter* strains

Azotobacter strains	N fixation (nmol C_2H_4/ml medium/h)
70	181.1
106	133.6
108	178.4

Table 12.5 N fixation capability of some *Azospirillum* strains

Azospirillum strains	N fixation (nmol C_2H_4/ml medium/h)
Al1	168
Al2	581
Al3	490
Al7	1139

Table 12.6 P solubilization capability of some *Bacillus* strains

Bacillus strains	P-solubilizing activity	
	Diameter of clear zone on agar medium (mm)	Available P concentration (mg/L)
B07	14.0	26.5
B04	20.0	39.3
B17	14.5	23.4
B19	15.5	25.0
Ba24	12.0	20.1
Ba34	13.0	22.0
Ba37	12.5	21.0

Table 12.7 Effect of N-fixing rhizobial strain on the growth of peanut

Treatment	N content of green biomass (gm/pot)	Plant height (cm)	Effective nodule (nodule/plant)	Dry green biomass (gm/pot)	Pod yield (gm/pot)
100 % NPK	1.55	60.4	185.9	81.8	25.50
100 % NPK+ rhizobial inoculation	2.02	67.0	220.0	89.8	28.56
90 % N + 100 % PK + rhizobial inoculation	1.98	66.1	216.0	88.4	27.82
80 % N + 100 % PK + rhizobial inoculation	1.80	63.5	207.1	86.8	27.61
70 % N + 100 %PK + rhizobial inoculation	1.59	62.6	200.6	83.5	26.38

research and development. Vietnam has national collection of agricultural beneficial microorganisms located at Soil and Fertilizer Research Institute and eight typical collection centers in different universities and research institutions. The national collection of agricultural beneficial microorganisms has more than 500 strains of bacteria, fungi, and streptomyces. Each year 30–50 new microbial strains are added to the repository (Nguyen Thu Ha 2015). The working model for isolation, selection, and preservation of microbial strains and bioinoculant production technology is summarized in Fig. 12.1.

Soil, water, and root samples are collected from fields, an isolation of beneficial microbes by plate count method on suitable growth media. The biological activities of microbes are measured by total N accumulation, 15 N dilution, and acetylene reduction assay (ARA) for nitrogen-fixing microbes; 32P isotopic techniques are employed for the determination of phosphate-solubilizing activity of microorganisms in the Pikovskaya's medium without agar (FNCA 2006). Tables 12.1, 12.2, 12.3, 12.4, 12.5, and 12.6 show the isolation results of some microbes as nitrogen-fixing and phosphate-solubilizing microorganisms in Vietnam.

Table 12.8 Effect of N-fixing *Azotobacter* on the growth of potato

Treatments	Plant height (cm)	% increase	Fresh green biomass (gm/plant)	% increase	Dry green biomass (gm/plant)	% increase
Control without inoculation	39.70	–	40.80	–	3.80	–
Inoculation with *Azotobacter*	42.50	7.0	43.60	6.9	4.20	10.5
NPK: 120.120.120	45.20	13.9	46.50	14.0	4.60	21.1
NPK: 96.120.120 + *Azotobacter* inoculation	45.30	14.1	46.60	14.2	4.65	22.4

Table 12.9 Effect of P-solubilizing *Bacillus* on the growth of potato

Treatments	Plant height (cm)	% increase	Fresh biomass (gm/plant)	% increase to control	Dry biomass (gm/plant)	% increase
Control without inoculation	39.7	–	40.8	–	3.80	–
Bacillus inoculation	42.8	7.8	44.0	7.8	4.30	13.2
NPK: 120.120.120	45.2	13.9	46.5	14.0	4.60	21.1
NPK: 120.60.120 + phosphate + *Bacillus* inoculation	45.1	13.6	46.5	14.0	4.59	21.0

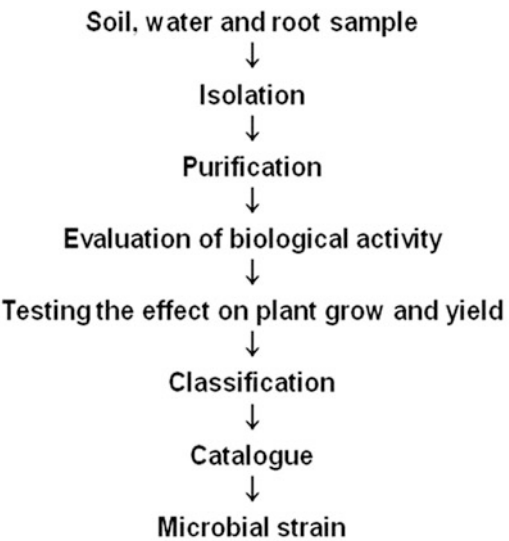

Fig. 12.1 Scheme of isolation and selection of agricultural important microorganisms

Table 12.10 List of beneficial microorganisms used as biofertilizers

Microorganism	Biological activity	Number of strains
Rhizobium	Symbiotic N fixation	242
Azospirillum	Associated N fixation	26
Azotobacter	Free living N fixation	36
Agrobacterium	Biocontrol	10
Arthrobacter	Plant growth promotion	12
Flavobacterium	Plant growth promotion	6
Serratia	Plant growth promotion	7
Klebsiella	Plant growth promotion	8
Exophiala	Plant growth promotion	1
Enterobacter	Free living N fixation	2
Bacillus	P solubilization	15
Pseudomonas	Antagonism to root pathogen	21
Candida	P solubilization	2
Trichoderma	Antagonism to root pathogen	18
Chaetomium	Nematicidal	1
Penicillium	Antagonism to root pathogen	2
Aspergillus	Antagonism to root pathogen	4

Isolates having high biological activities were tested for the growth, yield, and nutrition uptake of crops. Data in Tables 12.7 and 12.8 shows the N-fixing ability of rhizobia to form more nodules in groundnut as well as effects on growth and yield of tested crops in comparison to untreated control. Rhizobial-inoculated peanut fertilized with 30 % mineral N reduction has the same N content, green biomass, and pod yield as the control fertilized with 100 % required mineral N fertilizer.

P-solubilizing microbes inoculated to rice and vegetable have positive effects on growth, P uptake, and crop yield. Table 12.9 illustrates the effect of P-solubilizing *Bacillus* on the growth of potato. The results showed that the combination of *Bacillus* inoculation and rock phosphate fertilized with 50 % mineral production has the same effect on plant growth and green biomass as the control fertilized with 100 % required mineral P fertilizer.

The beneficial microbes used for biofertilizer research and development in Vietnam are listed in Table 12.10.

12.2.2 Development of Biofertilizer Production Technology

Different biofertilizers have been developed in Vietnam in the past. There are *Rhizobium* inoculants for legumes (soybean, groundnut); free living or associate N-fixing inoculant for rice, maize, and vegetables; P-solubilizing inoculants for agricultural and forestry crops; and multifunctional biofertilizer for industrial crops.

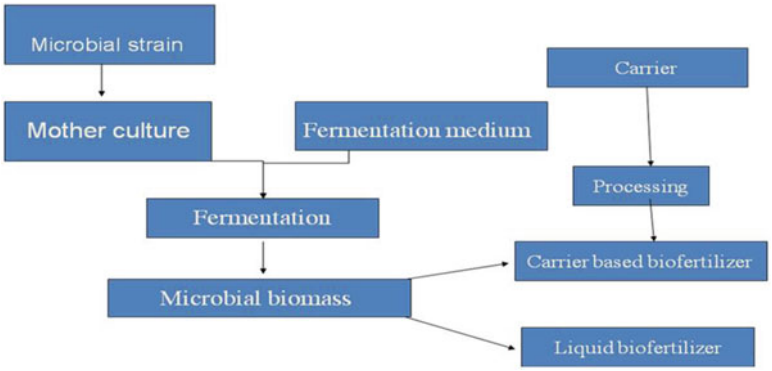

Fig. 12.2 Schema of carrier-based and liquid biofertilizer production

Table 12.11 Fermentation conditions of some beneficial microbes

Microbes	Optimal temperature (°C)	Optimal pH	Aeration (L air/L medium/minute)	% of start culture	Time of fermentation (h)
Rhizobium	28–30	6.8	5–10	5.0	48
Azotobacter	28	7.0	10	5.0	48
Azospirillum	28	6.8	5	5.0	48
Bacillus	28	7.0	10	5.0	36
Pseudomonas	30	7.0	10	5.0	36

Figure 12.2 illustrates the stages of carrier-based biofertilizer production in Vietnam. Microbial biomass is harvested from fermentation, treated to ensure the survival of microbes, and bottled as liquid biofertilizer or mixed to compost as organic biofertilizer.

For the biofertilizer production, selected microbial strains were tested for the growth and biomass production. All growth parameters like optimal growth medium, temperature, pH, oxygen concentration, and time of fermentation were evaluated. Table 12.11 shows the fermentation conditions of some N-fixing and P-solubilizing microbes in Vietnam.

Various types of material are used as carrier for inoculation. The carrier material should be milled to fine powder with particle size of 10–40 μm. Good carrier should have the following properties:

- Should be nontoxic
- Should have good moisture absorption capacity
- Should be easy to process and free of lump-forming materials
- Should be easy to sterilize by autoclaving or gamma irradiation
- Should be inexpensive
- Should have good pH buffering capacity

Table 12.12 Effect of irradiative dosage on the sterilization of peat carrier

Irradiated dosage (kGy)	Total aerobic (1000. CFU/g)	Total mold (1000 CFU/g)
0	200	+++
15	0.14	0.03
25	0.05	–
35	0.04	–
45	0.02	–

Table 12.13 Effect of different sterilizing methods on the survival of *Bradyrhizobium japonicum* in the inoculants

	Survival of *Bradyrhizobium* in the peat carrier-based inoculant in the storage of 10^8 CFU/g				
Sterilizing method	2 h	1 month	2 months	3 months	6 months
Control (non-sterilize)	2.5	24.0	0.1	–	–
Dry hot gas	27	26.0	25.0	20.0	10.5
Saturated steam	2.5	25.0	25.5	23.0	11.5
Gamma irradiation	2.4	40.0	45.0	47.0	37.0

Note: *Dry hot gas at 165 °C in 4 h and saturated steam of 1.5 Atm in 2 h*

In addition the carrier should ensure the survival of microbes during the storage period and survival of the inoculant bacteria in soil. In Vietnam peat is the most popular carrier material.

Different methods are used for the carrier sterilization. Effects of irradiation in the carrier processing and survival of *Rhizobium* can be seen in Tables 12.12 and 12.13. With the irradiation dosage of 25 kg, peat can be used as sterile carrier for the rhizobial inoculant (Table 12.12). The density of *Rhizobium japonicum* in the peat carrier based irradiated at 30 kg after 6 months of storage in polyethylene bags was 3.7×10^9/g, as compared with 1.05×10^9/g when sterilizing by dry hot gas and 1.15×10^9/g sterilizing by saturated steam (Table 12.13).

Liquid formulation of biofertilizers contains the desired microorganisms and nutrients along with the substances that encourage longer shelf life and tolerance to adverse conditions. The advantages of liquid biofertilizers over conventional carrier-based biofertilizers are the following: (a) longer shelf life (12–24 months), (b) no effect at high temperature, (c) no contamination and no loss of properties due to storage, (d) high populations can be maintained more than 10^9 cells/ml up to 12–24 months, (e) easy to use by the farmers, (f) high export potential, and (g) dosages are ten times less than carrier-based, quality control protocols that are easy and quick. The data in Table 12.14 shows the differences in the rhizobial density in solid and liquid formulation.

Table 12.14 Survival of *Bradyrhizobium* in liquid and solid formulations

Preservation time	Rhizobium strains	Rhizobium densities (CFU/g/ml)	
		Liquid inoculant with G5 medium	Peat carrier-based inoculant
0 h	132	5.00×10^9	1.32×10^9
	133	2.43×10^9	1.75×10^9
	57	1.28×10^9	9.40×10^8
2 weeks	132	6.20×10^9	1.00×10^9
	133	4.80×10^9	1.40×10^9
	57	1.46×10^9	8.70×10^8
1 month	132	5.60×10^9	2.54×10^9
	133	2.59×10^9	1.38×10^9
	57	9.80×10^8	1.89×10^8
2 months	132	3.12×10^9	1.81×10^9
	133	2.80×10^9	2.76×10^8
	57	8.10×10^8	6.06×10^8
3 months	132	2.15×10^9	1.56×10^9
	133	8.75×10^7	1.32×10^8
	57	9.40×10^7	1.00×10^8
6 months	132	1.39×10^8	1.33×10^8
	133	5.50×10^7	1.21×10^8
	57	2.26×10^6	1.20×10^8

Table 12.15 Effect of different inoculant formulations to the yield of soybean

Parameters	Winter season			Spring season		
	Type of inoculant			Type of inoculant		
	Solid	Liquid	No inoculation + 40 N	Solid	Liquid	No inoculation + 40 N
Nodule dry weight	1.389	1.347	0.766	2.761	2.706	2.169
Biomass dry weight	63.24	57.95	56.37	8.04	7.20	8.10
Seed dry weight	2.96	2.4.2	2.92	4.48	4.10	3.98

The effect of liquid biofertilizer on nodule formation and yield of soybean was tested, which showed no difference in the application of solid and liquid formulation (Table 12.15).

Multifunctional biofertilizers are the formulation containing consortia of N-fixing and P-solubilizing microbes and microorganism antagonistic to soilborne pathogens. Different multifunctional biofertilizers have been developed in Vietnam. The list of microbes used for multifunctional biofertilizer is presented in Table 12.16.

Effects of multistrain inoculant on target crops were tested in the greenhouse. The data in Tables 12.17, 12.18, and 12.19 showed that multistrain inoculant has positive effect on the growth of tested crops and is able to control the bacterial wilt

Table 12.16 Consortia of multifunctional biofertilizer

Target crops	Beneficial microbes	Biological activity	Amount
Peanut	*Bradyrhizobium* RA18	Acetylene reduction assay	144 µmol/plant/h
	Bradyrhizobium RA04	Indole acetic acid production	47.0 mg/l
	Bacillus Ge 67	Diameter of P-solubilizing zone	23.0 mm
	Pseudomonas Ps56	Diameter of inhibited zone	21.0 mm
Potato	*Azotobacter* AT03	Acetylene reduction assay	187.7 µmol/ml/h
	Bacillus B14	Diameter of P-solubilizing zone	20.0 mm
	Pseudomonas Ps56	Diameter of inhibited zone	20.0 mm
	Azotobacter AN 11	Indole acetic acid (IAA) production	15.3 mg/l
Tomato	*Azotobacter* AT03	Acetylene reduction assay	187.7 µmol/ml/h
	Azotobacter AT19	Indole acetic acid production	81.0 mg/l
	Bacillus B14	Diameter of P-solubilizing zone	20.0 mm
	Bacillus B16	Diameter of inhibited zone	16.0 mm

Table 12.17 The capability of multifunctional biofertilizer to control the bacterial wilt on tomato

	Dry green biomass	
Treatments	(g/plant)	% increase to control
Control	3.207	–
Inoculation with *R. solanacearum*	2.660	−17.16
Inoculation with multifunctional biofertilizer	4.760	48.43
Inoculation with *R. solanacearum* and multifunctional biofertilizer	4.330	35.02
CV%	8.2	
LSD	0.4606	

disease. Depending on the crop and location, the bacterial wilt control effect can be more or less than 90 %.

12.2.3 Effects of Biofertilizer on Crop Production

Biofertilizers were tested in field condition following the regulation of the Ministry of Agriculture and Rural Development in Vietnam. The effect of different biofertilizers is presented in Figures 12.3 and 12.4 showing that rhizobial inoculant can increase grain yield of groundnut by 13.8–17.5 % in North Vietnam and 22 % in South Vietnam. Experiments to evaluate the N fertilizer use efficiency showed that

Table 12.18 The capability of multifunctional biofertilizer to control the bacterial wilt on peanut

Treatments	Nodule number (nod/plant)	Dry green biomass	
		(g/pot)	% increase to control
Control	0.00	1.142	–
Inoculation with *R. solanacearum*	1.67	0.520	−54.47
Inoculation with multifunctional biofertilizer	32.54	1.578	38.18
Inoculation with R. *solanacearum* and multifunctional biofertilizer	31.12	1.509	32.14
CV (%)	–	10.50	
LSD	–	0.252	

Table 12.19 The capability of multifunctional biofertilizer to control the bacterial wilt on potato

Treatments	Dry biomass (g/plant)	% increase to control
Control	1.51	–
Inoculation with *R. solanacearum*	1.37	−9.27
Inoculation with multifunctional biofertilizer	2.20	45.69
Inoculation with R. *solanacearum* and multifunctional biofertilizer	2.09	38.41
CV(%)	5.5	
LSD	0.16	

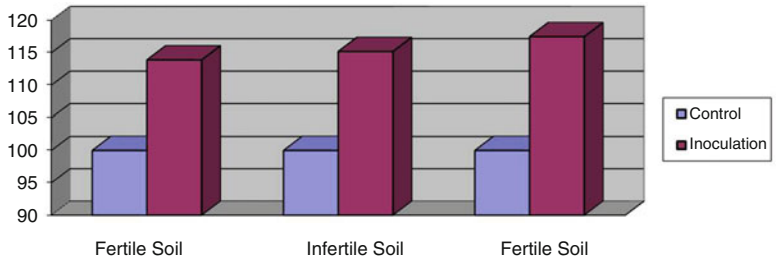

Fig. 12.3 Effect of rhizobial inoculant to increasing groundnut yield in North Vietnam (%)

rhizobial inoculant has the same effect like mineral N dose of 60–69 kg N/ha (Table 12.20). Thus, rhizobial inoculation can efficiently save 20–30 kg mineral N/ha.

The economical benefits of rhizobial inoculation are used in Table 12.21 and were calculated to about 442.000 VNĐ/ha.

N-fixing inoculant had positive effects on the growth and yield of rice. It depends on the fertilizer status and nutrition content of soil. It can increase the rice yield by 4.07–19.59 % or can save 20 % of required N fertilizer (Fig. 12.5).

Application of N-fixing inoculant to maize and tea can increase the yield and nutrient uptake in maize (Fig. 12.6) and tea (Fig. 12.7).

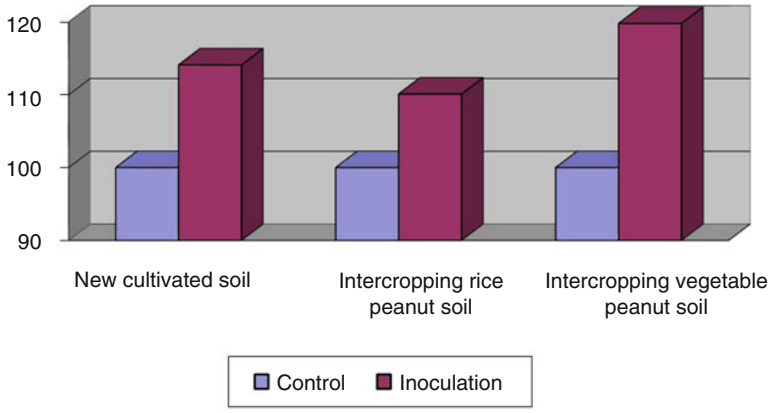

Fig. 12.4 Effect of rhizobial inoculant to increasing peanut yield in South Vietnam (%)

Table 12.20 Efficacy of rhizobial inoculation in peanut production

Treatments	Total pods (pod/plant)	Effective pods (pod/plant)	Yield (tons/ha)
Basal fertilizer (P60, K60, 8 t FYM, 400 kg lime) + 30 N	15.5	7.0	1.86
Basal fertilizer + 30 N + VKNS	16.9	7.5	2.05
Basal fertilizer + 60 N	16.9	7.2	1.85
Basal fertilizer + 90 N	18.2	6.9	1.91

Table 12.21 Economical benefit of rhizobial inoculation in peanut production

Treatments	Pod yield (tons/ha)	Green biomass (tons/ha)	Benefit (1000VND/ha)
No inoculation (control)	3.01	16	5.836
Inoculation	3.11	17	6.201
No inoculation + 30 N	3.22	18	6.429
Inoculation + 30 N	3.23	17	6.465
No inoculation + 100 N	3.19	18	5.857
SE	±51.5	±0.139	±197.500

Using the *Azospirillum* inoculation can save mineral N fertilizer. The data in Table 12.22 showed that *Azospirillum* inoculation is equivalent to application of 14.15–17.36 kg mineral N fertilizer depending on the soil types and growth season.

Azospirillum inoculant in maize and tea in Vietnam (Table 12.23) showed that *Azospirillum* inoculant can increase yield by 9.42–10.17 % in maize and 17.98 % in tea in comparison to the control without inoculation.

Phosphate-solubilizing biofertilizer is applied to rice and other food crops in Vietnam. Tables 12.24 and 12.25 showed the result on the effects of phosphate-solubilizing biofertilizer on the growth and yield of rice (Fig. 12.8).

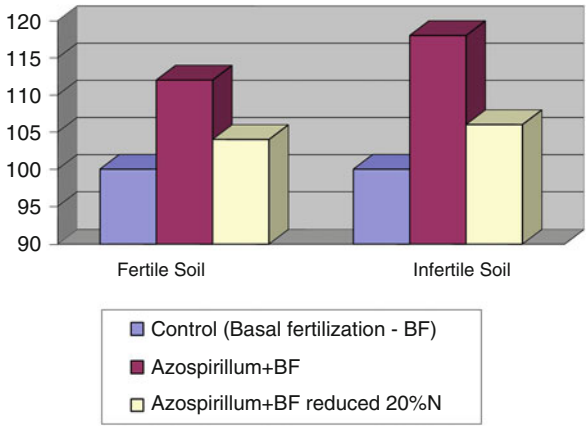

Fig. 12.5 Effect of *Azospirillum* inoculation to increasing the rice yield (%)

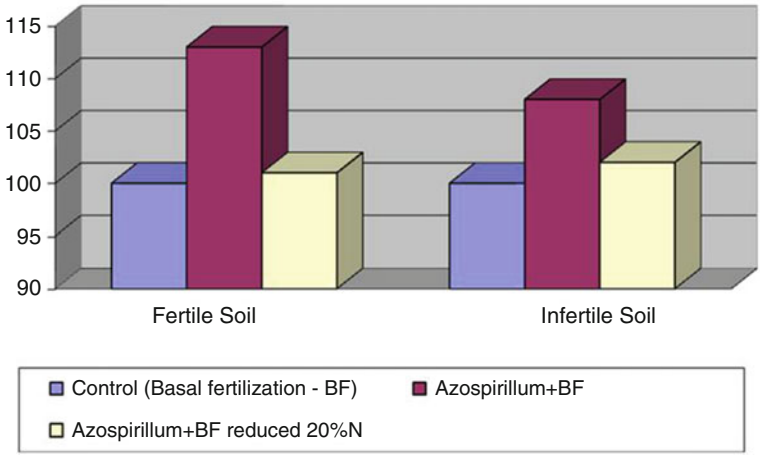

Fig. 12.6 Effects of *Azospirillum* inoculation to increasing the maize yield (%)

Using phosphate-solubilizing biofertilizer for potato can increase plant growth as well as biomass of tested crops (Table 12.25). The dates showed the combination of phosphate-solubilizing biofertilizer and rock phosphate can reduce 50 % required mineral P fertilizer.

N fixation has synergistic effect on P-solubilizing microbes. The results of studies on the synergistic effect of N-fixing and P-solubilizing biofertilizer on rice, soybean, and citrus (Tables 12.26 and 12.27) showed the effect on the growth and yield of different crops. Mixed inoculant can increase the yield of rice by 14.7–15.7 % in small experimental scale and by 10.65–12.7 % in large field trial (Table 12.26). Increasing by comparison without inoculation, inoculated soybean gives more yield of 16.3–19.5 % (Table 12.27). The same effect can be seen in the study on the citrus (Table 12.28).

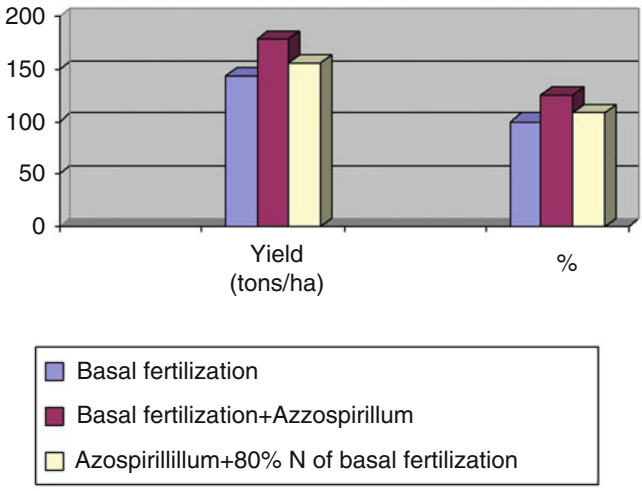

Fig. 12.7 Effects of *Azospirillum* inoculation on the tea yield

Table 12.22 N-fixing capability of *Azospirillum* on the rice

Soil types	N-fixing capability (kg N/ha)	
	Spring season	Autumn season
Fertile soil of Red River Delta	14.28	10.0
Fertile soil of Ma River	15.28	12.2
Infertile soil	22.40	16.0
Sandy soil	17.46	17.8
Average	17.6	14.5

Multifunctional biofertilizer was applied to tomato, peanut, and potato. The results of field testing in small experiment plot can be seen in Tables 12.29, 12.30, and 12.31. It can be concluded that multifunctional biofertilizer is able to increase crop yield and reduce 20 % of required mineral N and P fertilizer as well as reduce the bacterial wilt diseases in tomato, potato, and peanut.

Results of field demonstration on the effects of multifunctional biofertilizer on different crops in different locations are summarized in Table 12.32.

The economical effect of multifunctional biofertilizer on crop production (Table 12.33) showed that multifunctional biofertilizers are economically beneficial for farmers, who can have savings of 1.273 million VND to 13.850 million VND per ha.

Table 12.23 Effect of *Azospirillum* inoculant on the yield of maize and tea

Soil and crops	Fertilization	Yield (tons/ha)	% increasing to control
Maize on fertile soil	Control: NPK,180.120.90 + 10 t FYM (basal fertilization)	4.14	–
	Basal fertilization + *Azospirillum* inoculation	4.53	9.42
	80 % N of basal fertilization + *Azospirillum* inoculation	4.03	−2.58
Maize on infertile soil	Control: NPK, 180.120.90 + 10 t FYM (basal fertilization)	2.95	
	Basal fertilization + *Azospirillum* inoculation	3.25	10.17
	80 % N of basal fertilization + *Azospirillum* inoculation	2.87	−2.28
Tea on fertile soil	Control: NPK, 120.60.60 (basal fertilization)	14.29	–
	Basal fertilization + *Azospirillum* inoculation	16.86	17.98
	80 % N of basal fertilization + *Azospirillum* inoculation	15.10	5.66

Table 12.24 Effect of phosphate-solubilizing biofertilizer on the growth of rice

Parameters	Control without inoculation	P-solubilizing inoculation	% increase to control
Plant height (cm)	32.48	32.88	1.23
Effective panicle/hill	5.00	5.58	11.60
Dry green biomass (g/plant)	4.35	4.87	11.95
Total P of the plant (%)	0.25	0.32	24.00
Total N of the plant (%)	1.69	1.98	17.16

Table 12.25 Effect of phosphate-solubilizing biofertilizer on the growth of potato

	Fresh green biomass		Dry green biomass	
Treatments	(g/plant)	% increase to control	(g/plant)	% increase to control
Control without inoculation	40.8	–	3.80	–
P-solubilizing inoculation	44.0	7.8	4.30	13.2
NPK: 120.120.120	46.5	14.0	4.60	21.1
NPK: 120.60.120 + phosphorite + P-solubilizing inoculation	46.5	14.0	4.59	21.0

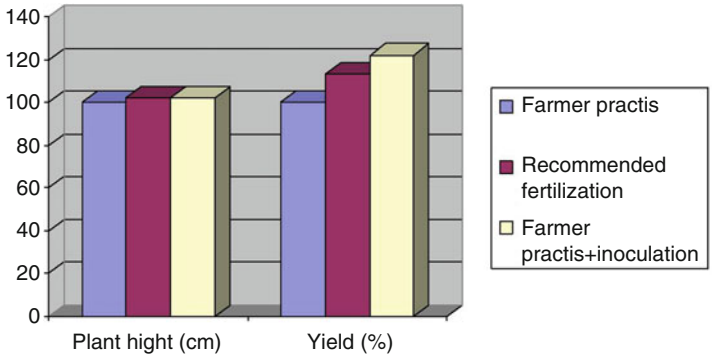

Fig. 12.8 Effect of P-solubilizing inoculation to increasing the plant height and yield (%) of maize

Table 12.26 Effect of mixed inoculant on rice production

Treatments	Experiment in small scale		Profit (1000 VND)	Experiment in large scale	
	Yield (tons/ha)	% increase to control		Yield (tons/ha)	% increase to control
Spring season					
Control	3.81	–	1683.9	3.61	
Inoculant	4.39	15.2	2755.2	3.99	10.73
LSD	3.24				
Autumn season					
Control	4.28	–	4348.4	4.15	–
Inoculant	4.95	15.7	4716.6	4.67	12.52
LSD	5.25				

Control: Spring season 100 N +70P_2O_5 + 30K_2O
Autumn season 90 N+ 45 P_2O_5 + 30K_2O
Inoculation: Spring season 80 N + 25 K_2O+ 526 kg RP + mixed inoculant
Autumn season 70 N + 25 K_2O + 526 kg RP + mixed inoculant

12.2.4 Biofertilizer Production and Application in Vietnam

Biofertilizer research and development began in Vietnam more than 20 years ago, but the production and application of biofertilizer are limited. There is no factory producing sterile inoculant in the country. They are produced only by research organizations in small scale. In general peat is used as carrier and sterilized by autoclave before it is inoculated with microbial biomass. Radiation sterilization technique is applied, but only for the research purpose and at laboratory scale. This kind of biofertilizer has good quality, but lacking large-scale production facility, information, and demonstration, their application is mostly in the experiment of some research project. The inoculant using area changes from many thousand to hundred thousand hectares per year, depending on the budget of the project. Most of biofertilizers

Table 12.27 Effect of mixed inoculant on soybean production

Treatments	Trial in small scale			Trial in large scale	
	Yield (tons/ha)	% increase to control	Profit (1000 VND)	Yield (tons/ha)	% increase to control
Control	19.0	–	6160.8	19.00	–
Inoculant	22.7	19.5	7865.7	21.58	13.58
LSD	2.31	–		–	–

Control: 25 N+ 45 P_2O_5 + 30K_2O
Inoculation: 20 N + 40 K_2O + 550 kg rock phosphate + mixed inoculant

Table 12.28 Effect of mixed inoculant on citrus production

Treatments	Yield (tons/ha)	% increase to control	Economical efficiency (1000 VND)		
			Output	Input	Profit
Control	10.95	–	32,850	19,614	13,236
Inoculant	12.30	1.3	36,900	19,296	17,640
LSD	0.4				

Control: 27,500 kg FYM + 500 kg lime + 177 N + 272 P_2O_5 + 132 K_2O
Inoculation: 3300 kg rock phosphate + mixed inoculant +140 N + 105K_2O

Table 12.29 Effect of multifunctional biofertilizer on potato production

Treatments	Yield		Bacterial wilt	
	tons/ha	% increase to control	%	% decrease to control
Control (basal fertilization) 120 N, 70P, 90 K + 20 t FYM	13.8	–	23.4	–
Basal fertilization + multifunctional biofertilizer	16.2	17.39	9.4	59.83
Basal fertilization reduced 20 % of N and P + multifunctional biofertilizer	14.6	5.79	10.8	53.85
Basal fertilization reduced 300 % of N and P + multifunctional biofertilizer	14.5	5.07	12.0	48.72
LSD	5.39			

applied in Vietnam use non-sterile inoculant. It is produced by mixing microbial mass and ripped compost with simple technique. Their quality is low and their effect on plant growth and yield is irregular. In Vietnam there are more than ten companies that produce 20 types of biofertilizer.

12.3 Regulatory Requirements of Biofertilizer in Vietnam

At present, Vietnam does not away law directly for biofertilizer production and commercialization. Indirectly there are laws on standardization and technical regulation; the Law on Quality of Commercial Products, 2008, regulates the production

Table 12.30 Effect of multifunctional biofertilizer on tomato production

Treatments	Yield		Bacterial wilt	
	tons/ha	% increase to control	%	% decrease to control
Control (basal fertilization) 120 N, 70P, 90 K + 20 t FYM	16.85	–	–	–
Basal fertilization + multifunctional biofertilizer	19.50	19.27	15.73	75.00
Basal fertilization reduced 20 % of N and P + multifunctional biofertilizer	18.89		12.11	68.85
Basal fertilization reduced 300 % of N and P + multifunctional biofertilizer	18.35	11.35	8.90	67.29
LSD	2.6			

Table 12.31 Effect of multifunctional biofertilizer on peanut production

Treatments	Yield		Bacterial wilt	
	tons/ha	% increase to control	%	% decrease to control
Control (basal fertilization) 30 N, 90P, 60 K + 10 t FYM	2.02	–	50.0	–
Basal fertilization + multifunctional biofertilizer	2.24	10.84	37.4	25.2
Basal fertilization reduced 20 % of N and P + multifunctional biofertilizer	2.21	9.20	38.8	22.4
LSD	2.6			

and commercialization of biofertilizer. Setting up the laws, the Vietnam government approved the Decree No. 89/2006/NĐ-CP on 30 August 2006 that requires the labeling of commercial products; the Decree No. 127/2007/NĐ-CP on 01 August 2007 to implement the Law on Standardization and Technical Regulation, 2006; the Decree No.15/2010/NĐ-CP on 01 March 2010 to regulate the administrative sanctions in fertilizer production and commercialization; and the Decree No. 202/2013/NĐ-CP on 27 November 2013 to regulate the fertilizer management. Figure 12.9 summarizes the system of fertilizer management in Vietnam. Regarding Decree No. 202/2013/NĐ-CP, the Ministry of Agriculture and Rural Development (MARD) is responsible for organic and other fertilizers including biofertilizer production and commercialization in Vietnam. On 13 November 2014, MARD approved the Regulation No. 41/2014/TT-BNNPTNT to implement the Decree No. 202/2013/NĐ-CP belonging to the responsibility of MARD. It contains the general regulation on the production and commercialization of biofertilizers; the permit for production, trade, import, and export; the quality management; the field trial; and the responsibility of management agencies of MARD, Department of Crop Production of provinces, and fertilizer producers and traders.

Table 12.32 Effect of multifunctional biofertilizer on crop yield and root disease control in large-scale field trials

Crops and location	Yield (tons/ha)		% increase	% root disease		% decrease to farmer practice
	Farmer practice	Biofertilizer		Farmer practice	Biofertilizer	
Tomato – Vinh Phuc Province	15.52	1.85	19.27	1.25	0.00	100
Potato – Hanoi Province	12.9	14.50	11.24	24.6	11.3	55.1
Peanut – Namdinh Province	2.13	2.52	16.73	40.0	15.0	62.5
Cabbage – Namdinh Province	27.15	30.72	13.15	–	–	–
Watermelon – Hoa Binh Province	17.5	20.00	13.0	12.0	3.3	72.5
Pepper – Dak Nong Province	2.28	3.50	53.51	6.7	4.7	29.99
Cotton – Dak Lak Province	1.48	1.86	25.96	8.6	0	100
Coffee – Dak Lak Province	5.67	6.84	20.63	–	–	–

Table 12.33 Economical effect of multifunctional biofertilizer to crop production (1000 VND)

Crops	Fertilizer input		Benefit to farmer practice		
	Farmer practice	Biofertilizer	From yield increase	From decreasing the input	Total benefit
Tomato	6127	5527	6460	600	7060
Potato	5830	5630	4800	200	5.000
Peanut	3183	2383	3120	800	3920
Cabbage	6105	4905	7140	1200	8340
Pepper	9790	7790	4270	2000	6270
Cotton	1499	1967	1742	−468	1273
Coffee	7780	11,560	17,550	−3700	13,850

General quality requirements of biofertilizer in Vietnam are as follows:

- Density of beneficial microbes $\geq 10^8$ CFU/g(ml) (biofertilizer) and $\geq 10^5$ CFU/g (organic biofertilizer)
- Shelf life ≥6 months
- Biosafety for human, animal, and environment (microbes and carrier or additives)
- Positive impact to plant growth, yield, product quality, or nutrition uptake by the plant

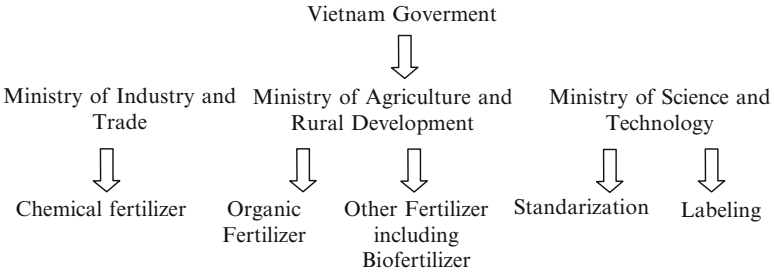

Fig. 12.9 Management system for the fertilizer production and commercialization in Vietnam

The registration of biofertilizer in Vietnam should follow three steps:

- Conduction of the field trial to evaluate the impact of biofertilizer on the growth, the yield of crops, and the effectivity of tested fertilizer
- Declaration of biofertilizer to satisfy the national technical regulation and focus on the main characters, the quality, and the application guideline of biofertilizer
- Acceptance of the declaration by the provincial Department of Crop Production and Department of Crop Production of MARD

12.4 Conclusion

Biofertilizer has positive effect on plant growth and yield of most agricultural crops in Vietnam. The research program of inoculant is well established. But its production and application are limited. Problems and solutions can be faced as follows:

– System of research from pilot to industrial production is slow and expensive; therefore, the research results cannot be tested and applied in the practical production. Thus, establishing a demonstration model of biofertilizer production technology from laboratory scale to production scale will help to scale up the use of biofertilizers in Vietnam.
– Low quality of biofertilizer negatively affects the farmers. New technology and techniques should be developed and transferred for production. Multifunctional inoculant is one of the priorities in future research. On the other hand, there is an urgent need to establish the system of biofertilizer users.
– On farm demonstration and training programs for extension workers and farmers can improve the knowledge and experience of the biofertilizer user.
– International cooperation will improve the current status of biofertilizer research, development, and application in Vietnam.

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Biopesticides Research: Current Status and Future Trends in Sri Lanka

13

R.H.S. Rajapakse, Disna Ratnasekera, and S. Abeysinghe

Abstract

Sri Lanka is a tropical country equally having rich diversity of arthropods including natural enemies, economic pests, and indigenous plants majority with unique chemical properties. Because of the substantial losses due to pests and diseases, plant protection remains an essential issue in agriculture production in the country. There is increasing concern over synthetic pesticide usage due to their adverse long-term effects on human health, environment, and natural pest management systems. As an eco-friendly alternate, the importance of biopesticides in raising agricultural productivity is well recognized in Sri Lanka. Biopesticides are quiet popular among farming community due to their unique features, viz., safety, limited host range or target specificity, the absence of toxic residues, eco-friendly nature, and ease of application. Biopesticides have diverse modes of action and hence resistance development in pests is slower/negligible. Currently, plant powders, nonvolatile and volatile oils, and plant crude extracts are commercially available for management of insect pests and nematodes. Further, several bacterial and fungal biopesticides have shown promising results for the efficient management of plant pathogens in Sri Lanka.

Keywords

Biopesticides • Biocontrol agents • Organic farming • Agriculturally important microorganisms

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13.1 Introduction

Sri Lanka has been known as an agricultural country since ancient times. Approximately 30 % of the 6.5 million ha of land area in Sri Lanka are under cultivation. Due to the substantial crop losses caused by pests and diseases, plant protection remains an essential issue for improving agriculture production in the country. The present annual synthetic pesticide consumption is estimated at 1700 t of active ingredients amounting to approximately Rs 4.6 billion. There is an increasing concern over synthetic pesticide usage due to their adverse long-term effects on human health, environment, and natural pest management systems. As an alternate, the importance of biopesticides in raising agricultural productivity is well recognized in Sri Lanka.

The term biopesticides refer to those pesticides obtained from biological sources such as microbes (fungi, bacteria, viruses, protozoa, and nematodes), semiochemicals (insect sex pheromones), biochemicals (substances from biological sources especially botanicals such as essential oils, nonvolatile oils, and extracts), and commercially produced natural enemies (predators and parasitoids). Biopesticides are distinguished from synthetic pesticides by their unique features: safety, limited host range or target specificity (very low risk to nontarget organisms), the absence of toxic residues on fruit and vegetables (easily degradable in nature), environment-friendly, and easily applied using conventional spray equipment. Biopesticides offer diverse modes of actions; hence, resistance development in pests is slower. Therefore, there is immense scope in identifying and developing biopesticides as alternative pest management strategies. The importance of novel and alternative biopesticides and the necessity for research and development of novel, cost-effective, environmentally friendly pesticide is well recognized in Sri Lanka.

Limitations and challengers of commercializing biopesticides are slow action and low persistence when exposed to solar UV, high cost of production, and lack of awareness among Sri Lankan farmers. The major constraints to biopesticide development are poor awareness of decision-makers about opportunities offered by biopesticides, lack of multidisciplinary expertise in the crucial later stages of development, difficulty in conducting toxicological tests, and the long testing period of bioactive compounds before registration and commercialization.

13.2 Classification of Biopesticides

Biopesticides are broadly categorized into two groups, biochemical pest control agents and microbial pest control agents.

Biochemical pest control agents: these chemicals are not directly toxic to target organisms like nerve poisons and exhibit different mode of action like mating disruption, molt inhibition, and growth regulation. They are naturally occurring substances; if synthesized, they must be identical to the natural chemical. There are four classes of compounds that fall into biochemical pest control agents:

1. Semiochemicals: pheromones, allomones, and kairomones
2. Hormones: molt hormones (ecdysteroids) and juvenile hormones (IGR)
3. Natural plant regulators: auxins, gibberellins, cytokinins, and inhibitors
4. Enzymes

Microbial pest control agents: these include formulations that occur in nature or organisms effective as pest management agents. Microbial pest control agents include:

1. Bacteria: *Bacillus thuringiensis* (Bt)
2. Fungi: *Verticillium*, *Metharizium*, and *Hirsutella*
3. Virus: *Nuclear polyhedrosis virus* (NPV)
4. Nematodes: *Steinernema* sp.

13.3 Biopesticides Used in Agriculture in Sri Lanka

Nearly 30 % of the 6.5 million ha of lands in Sri Lanka are cultivated to agricultural crops. Of this, the food crop sector dominated by rice (730,000 ha), vegetables (90,000 ha), root and tuber crops (100,000 ha), fruits, other field crops, and export agricultural crops (200,000 ha) occupies around 12 million ha. The plantation crops and other perennials occupy around 700,000 ha. Sri Lanka has a very rich natural enemy complex of crop pests as compared to tropical Asian countries giving more opportunities for the utilization of natural biocontrol. For instance, the egg parasitism of brown plant hopper found to reach as high as 80 % in fields kept free of pesticides during the early vegetative stage. Furthermore, rice land spiders fauna were found very effective in managing hopper pests and also less affected by diamondback moth damage when the wrapper leaves are kept free of insecticides to allow the multiplication of parasitoids like *Cotesia plutellae*, *Diadegma* sp. The leaf miner, *Liriomyza* sp., damage found to be low when the affected crops are sprayed only with neem extracts as compared to other insecticides. These evidences suggest the vast potential available for the application of biological pest control agents for crop pest management in Sri Lanka (Table 13.3).

However, at present, only a few pesticides that qualify as biological pesticides are marketed in Sri Lanka (Tables 13.1 and 13.2). Furthermore, farmer acceptance and applicability of these products at field level are found to be very low. This could be due to the inherent features of the biopesticides that require careful planning in the treatment schedules than the conventional pesticides and the low profile given for promotional activities including education of farmers and extension staff.

Table 13.1 Commercially available biopesticides and other related compounds in Sri Lanka

Common name	Origin	Trade name/mode of action	Toxicity	Remarks
Bacillus thuringiensis Bt	*Bacillus thuringiensis* subsp. *kurstaki*	Bt 85 % WG	Stomach poison. The endotoxin crystals are solubilized, and the epithelial cells of the gut are damaged, insects stop feeding, and eventually starve to death	Acute oral LD50 for rats > 2.67 g/kg, 1 × 10^{11}spores/kg
Abamectin	Isolated from fermentation of *Streptomyces avermitilis*. Avermectin (Vertimec)	Acts by stimulating the release of r-aminobutyric acid, an inhibitory neurotransmitter, thus causing paralysis		Acute oral LD50 for rats 10s
Azadirachtin/neem seed water extract	Principal insecticidal ingredient of neem seed extract – contains limonoids	Neemasal and Neemgrow	Ecdysone antagonist. Disrupts insect molting. Fungicidal and miticidal, antifeedant and repellent	Acute oral LD50 for rats >5000 mg/kg

13.4 Regulatory Frameworks and Research and Training in Pest Management

13.4.1 Botanical Products

Many plant species with pest control properties were identified and used at village level in small scale, and Sri Lankan farmers have sound knowledge about the indigenous practices of utilization of plant products for pest management. Neem products are highly utilized in pest management, and many researches have been conducted to estimate their possible effects with fulfilled results. A number of neem-based commercial products are available in Sri Lankan market for agricultural uses in different trade names, and it is recommended to use for the control of

Table 13.2 Commercially available insect growth regulators, chitin synthesis inhibitors, molt-accelerating compounds in Sri Lanka

Common name	Origin	Trade name	Mode of action	Toxicity	Remarks
Tebufenozide	Molt-accelerating compound	Mimic	Ecdysone agonist, binds to the receptor sites of molting hormone, ecdysone, lethal molting	Acute oral LD50 – rats 5000 mg/Kg	Recommended for the control of leaffolder of rice and leaf-eating caterpillar on vegetables
Methoxyfenozide	Molt-accelerating compound	Runner	Accelerate molting	Acute oral LD50 – rats >5000 mg/Kg	Recommended for the control of leaffolder of rice and leaf-eating caterpillar on vegetables
Chlorfluazuron	Chitin synthesis inhibitor	Atabron	Anti-molting agent	Acute oral LD50 – rats 5000 mg/Kg	Recommended for the control of leaffolder of rice and leaf-eating caterpillar on vegetables
Cyromazine	Inhibits molting and pupation	Trigard	IGR with contact action interferes with molting and pupation systemic in plants	Acute oral LD50 – rats 3387 mg/Kg	Effective against leaf miners
Buprofezin	Chitin synthesis inhibits	Applaud	Chitin synthesis and prostaglandin inhibition	Acute oral LD50 – rats 2000 mg/Kg	Recommended against BPH and whiteflies
Novaluron	Chitin synthesis inhibits	Rimon	Affect molting, abnormal endocuticular deposition, and abortive molting	Acute oral LD50 – rats 5000 mg/Kg	Recommended against BPH, fruit borer, and leafhopper

leaf-eating caterpillars in vegetables, diamondback moth in cabbage, rice leaffolder, sesame leaf webber, leaf miners in vegetables, and mites in vegetables. In addition, neem-based pesticides have a considerable potential for controlling *Callosobruchus* spp. (Rajapakse 1990) and *Sitophilus oryzae* (Mannakkara 2002) under storage conditions. The other popular botanical products for pest management are *Allium*

Table 13.3 Biopesticides with potential use in Sri Lanka for crop pest management

Common name	Origin	Trade name	Mode of action	Toxicity	Remarks
Spinosad (spinosyn C and D)	Actinomycetes *Saccharopolyspora spinosa*	Success	Nicotinic acetylcholine receptor, different from nicotine or imidacloprid leads to paralysis	Acute oral LD50 – rats 5000 mg/kg	No residues, rapidly degraded, for leaf miner control
Metarhizium anisopliae local cultures	Entomopathogenic fungus	–	–	LC50 for rats > 4850 mg/m^3	BPH control in rice
Predatory mites	–	–	–	–	For the control of coconut mites
Spodoptera exigua NPV	Baculoviridae: Nucleopolyhedrovirus	"Spod-X" Ness A WA, LC	Active by ingestion. Caterpillars cease feeding after 4 days and die after 5–10 days	–	For vegetable caterpillar control
Essential oils of plants like- *Cinnamomum zeylanicum*, *Cymbopogon nardus*, *C. citratus*, etc.	Bioactive compounds	–	Act as a repellents, growth inhibitors	–	Stored grain pests of rice, cereals, legumes, and potatoes

sativum, *Capsicum frutescens*, *Gliricidia sepium*, *Adathoda vesica*, *Pleurostylia opposita*, *Acronychia pedunculata*, *Alseodaphne semecarpifolia*, etc.

13.4.2 Plant-Derived Biochemicals Used in Postharvest Storage

The postharvest losses and quality deterioration caused by storage pests are major problems throughout the world and more vulnerable in tropical countries like Sri Lanka. Traditional methods of pest control in grains by mixing them with neem, citrus and maduruthala leaves, plant oils, and powdered plant materials have been utilized by farmers for many years.

Continuous research has been performed systematically to evaluate plant-derived biochemicals using various indigenous plant species in different forms such as

crude ethanol (CE) extracts, vegetable oils, dry powders, and combinations of plant materials with insecticides against *Callosobruchus* spp. in laboratory conditions (Rajapakse et al. 1998, 2002; Rajapakse and Ratnasekera 2009; Ratnasekera and Rajapakse 2012).

13.4.3 Crude Extracts

Extracting plant materials with an appropriate solvent generally results in concentration of active ingredients. Such extracts are therefore often more effective against storage beetles than powders of fresh plants. Usually the extracts are mixed with the seeds as a liquid, and the solvent evaporates before the seeds are stored. Many local plant species have been tested for bioactive compounds, and various degrees of bioactivities were recorded. According to Rajapakse and Ratnasekera (2008), the highest bioactivity (90–100 % mortality) was manifested by the crude ethanol extracts of *Azadirachta indica* (neem), *Annona reticulata* (anona), and *Ocimum sanctum* (maduruthala/sacred basil) among the 20 plant crude ethanol extracts tested. Extracts of *Myristica fragrans*, *Gliricidia sepium*, *Ricinus communis*, *Cajanus cajan*, *Mangifera indica*, *Eupatorium odorantum*, *Dioscorea polygonoides*, and *Hibiscus rosa-sinensis* showed no toxicity, while those of *Citrus reticulata*, *Artocarpus heterophyllus*, and *Cassia occidentalis* had little toxicity. *Capsicum annuum* and *Dillenia retusa* plant extracts were slightly toxic. *C. frutescens* and *Piper nigrum* were moderately toxic, while those of *Eugenia caryophyllata* caused fairly high-toxic plants to the beetle mortality (Table 13.1). Plant extracts of*Allium sativum*, *Piper guineense*, and *Capsicum annuum* from different solvents such as petroleum ether and ethanol effective on stored beetle adults are either acting as repellent, toxicant, or combination of these two actions (Rajapakse 2000).

The disadvantages of using crude extracts are mostly difficulty to prepare and laborious to make low yields and hence need large quantities of plant materials, and farmers have no facilities to extract plants in village level (Table 13.4).

13.4.4 Nonvolatile Oils

Mixing plant oils with stored seeds is common among farmer communities. Nonvolatile oil is used as a coating for seeds and effectively protects seeds against insect pests during stage. The film of oil prevents the attachment of the egg to the seed coat. Most of the oils are very effective and retain their effectiveness over a long period. The non-bitter taste of plant oil used is an added advantage over neem oil, which is known for its bitter taste. The relatively small amounts of oils required their effectiveness, and the simple technology of extraction will make these plant oils a better candidate for seed dressing purposes for cowpea storage. Nonvolatile oils can have negative effects on adult beetles through contact toxicity or through deterrence. With increase of prevailing prices of insecticides, the application of plant oils would be an inexpensive control method against *C. maculatus* and *C.*

Table 13.4 Toxicity of the ethanol extracts of the leaves of 20 plants to *C. maculatus* and *C. chinensis*

	Corrected mortality					
	Day-1		Day-2		Day-3	
Plant species	*C.c.*	*C.m.*	*C.c.*	*C.m.*	*C.c.*	*C.m.*
Capsicum frutescens	35 ± 1.0	40 ± 2.6	45 ± 1.2	48 ± 3.1	60 ± 6.6	60 ± 6.7
Myristica fragrans	00	00	00	00	00	00
Piper nigrum	35 ± 1.2	40 ± 1.8	48 ± 2.1	50 ± 2.7	55 ± 2.1	60 ± 6.5
Citrus reticulata	10 ± 0.8	12 ± 1.0	10 ± 1.0	08 ± 0.7	12 ± 1.1	14 ± 1.0
Cymbopogon citratus	50 ± 3.5	60 ± 5.2	50 ± 4.6	60 ± 4.3	65 ± 3.7	67 ± 4.9
Artocarpus heterophyllus	05 ± 0.5	08 ± 0.7	12 ± 1.0	11 ± 1.0	15 ± 1.1	17 ± 1.1
Gliricidia sepium	00	00	00	00	00	00
Eugenia caryophyllata	50 ± 4.8	60 ± 5.1	60 ± 4.8	60 ± 4.0	71 ± 5.6	70 ± 6.2
Ricinus communis	00	00	00	00	00	00
Dillenia retusa	40 ± 2.8	44 ± 3.0	50 ± 3.7	56 ± 3.8	50 ± 4.2	58 ± 4.7
Azadirachta indica	50 ± 3.5	60 ± 4.2	60 ± 4.0	65 ± 4.6	80±	6.7
Cajanus cajan	00	00	00	00	00	00
Cassia occidentalis	06 ± 0.4	09 ± 0.6	12 ± 0.8	13 ± 1.1	15 ± 1.3	18 ± 1.5
Annona reticulata	80 ± 5.7	80 ± 5.6	80 ± 6.3	90 ± 6.0	90 ± 6.8	91 ± 7.1
Mangifera indica	00	00	00	00	00	00
Eupatorium odoratum	00	00	00	00	00	00
Ocimum sanctum	80 ± 5.7	80 ± 5.6	80 ± 6.3	90 ± 6.0	90 ± 6.8	91 ± 7.1
Capsicum annuum	30 ± 1.8	35 ± 2.1	43 ± 3.8	50 ± 4.6	48 ± 3.9	45 ± 3.2
Dioscorea polygonoides wild	00	00	00	00	00	00
Hibiscus rosainensis	00	00	00	00	00	00

Source: Rajapakse and Ratnasekera (2008)

chinensis. Oils of *O. sanctum* at 1.5 μL and *A. reticulata* at 3.0 μL completely inhibited oviposition and adult emergence of *C. maculatus* and *C. chinensis* inferring their potential utilization (Ratnasekera and Rajapakse 2012). Oviposition could be influenced by treatment of the stored product with nonvolatile oil. Rajapakse and Vanemden (1997) reported that the four oils; corn, groundnut, sunflower, and sesame reduced oviposition of all three bruchid species tested. Further, groundnut, coconut, and soybean oils reported to be reduced adult mortality significantly compared to standard chemical control by pirimiphos-methyl. However, direct mixing oils might reduce consumer demand (Table 13.5).

13.4.5 Volatile Oils

Volatile oils can be extracted mostly by aromatic plants. The yield volatile oil is usually low, but due to repellence or toxicity, even small amounts of the concentrated essential extract can be very effective in airtight or hermetic storage

Table 13.5 The effect of standard chemical control, pirimiphos-methyl, and nonvolatile vegetable oil against *C. maculatus* adults

Treatments	Dosage	Mean (1 DAT)	% mortality (7 DAT)
Control		0.00[g]	2.700[g]
Pirimiphos-methyl	Full dose	100.00[a]	100.000[a]
	¾ dose	90.367[c]	95.300[b]
	½ dose	73.267[e]	85.700[e]
	¼ dose	65.000[f]	81.000[f]
Groundnut oil	10 ml/kg	95.000[b]	85.700[e]
Coconut oil	10 ml/kg	91.000[c]	92.000[c]
Soybean oil	10 ml/kg	85.000[d]	87.700[d]

Source: Rajapakse 2002

structures. Nayanathara and Ratnasekera (2010) reported that volatile fumes of cinnamon and citronella oil could effectively repel stored pests in bulk storages. According to their findings, no losses on viability and no off-flavors are detected. Hence, volatile oils have broad consumer preference in seed storage both for consumption and store as planting material as oils are not mix with seeds.

Further, the repellent effects of ten oils, Domba (*Calophyllum inophyllum* L.), Batu (*Solanum indicum* L.), leaf oil and bark oil of cinnamon (*Cinnamomum verum* Presl.), mustard oil (*Brassica juncea* Cross.), neem oil (*Azadirachta indica* A. Juss.), mee oil (*Madhuca longifolia* Koenig.), castor oil (*Ricinus communis* L.), citronella oil (*Cymbopogon nardus* L.), and sesame oil (*Sesamum indicum* L.) were tested for pulse beetle (*Callosobruchus maculatus* L.) in the laboratory conditions, and they reported that citronella oil, neem oil, cinnamon leaf oil, and cinnamon bark oil vapors showed significantly control in pulse beetles after infestation indicating the highest repellent action and toxic effects (Rajapakse and Ratnasekera 2009).

13.4.6 Plant Powders

Mixing plant materials as powder form is a simple technique that can be easily adopted among farmers. Many plants have been tested in the laboratory as powders to evaluate their possible effects. Clove powder was the most effective among the four powders tested for adult mortality followed by root dust of papaya. Among the plant powders tested, maduruthala (*O. sanctum*) was the most effective for suppressing oviposition significantly followed by geta thumba (*Leucas zeylanica*). Same study reported that enhanced toxicity and mortality to *Callosobruchus* spp. persistence of the insecticide in causing significant reduction when combined with vegetable oils. Further, these results revealed the potential applicability of some indigenous plant materials as stored grain protectants. The modes of action of these substances are not yet known, and further studies must be carried out especially to clarify how it is involved in the physiology of reproduction.

13.5 Microbial Pest Control Agents

Currently, many scientists are focusing research on utilization of microorganisms as a biofertilizer, and several studies have been done to identify the advantages of utilizing *Rhizobia*, *Azotobacter*, *Azospirillum*, blue-green algae, *Azolla*, and phosphate solubilizers (several bacteria and fungi) in local conditions, and it showed good results.

The soil is the natural habitat of N-fixing bacteria, but due to the degradation of agricultural soil, often our soil do not have either the proper kind of nodule-forming bacteria or enough quantity of certain bacteria to enhance the good legume growth. *Rhizobium* is the most well-known species for symbiotic nitrogen fixation, and many research works have been done to the collection, isolation, and subsequent selection of effective rhizobial strains, and its uses in agriculture have yielded fruitful results. And also, use of *Rhizobia* as biofertilizers in mushroom cultivation also seems to be a promising result in experimental basis (Senevirathna et al. 2009).

Utilization of *Azolla* spp. for nitrogen fixation in paddy land results strong support to the potential use as a biofertilizer for rice in Sri Lanka. Seventy-three strains of nitrogen-fixing blue-green algae, belonging to 21 genera, were isolated in rice soils in Central Sri Lanka (Kulasooriya and de Silva 1981).

The basic research on P-solubilizing biofertilizers was successfully established resent past. Biosolubilization of rock phosphate using fungal solubilization such as a *Penicillium* spp., an *Aspergillus* spp., *Pleurotus ostreatus*, *Bradyrhizobium elkanii*, and P-solubilizing bacteria was investigated and identified an effective method of fungal-rhizobial biofilm-mediated solubilization of Eppawala rock phosphate deposit in Sri Lanka (Jayasinghearachchi and Seneviratne 2006).

Plantation sector of Sri Lanka faces massive problem on soil degradation due to mismanagement of soil resources and monoculture cropping system adopted in plantation sector. To minimize this target, usage of inorganic and organic manures and biofertilizer will have a major and important role to play. Basic research has been implementing studies on biofertilizers based on microbial biofilms and their effects on soil carbon accumulation. In a nursery tea soil, a liquid formulation of biofilmed biofertilizers together with 50 % of recommended chemical fertilizers for tea increased soil organic carbon by 30 %, compared to the application of 100 % of the recommended fertilizers alone (Jayasekara et al. 2008). Many basic researches have been done to utilization of mycorrhizal inoculants to improved crop productivity and showed that inoculation of tea cuttings with VAM significantly enhanced the growth of tea seedlings.

13.5.1 Bacteria

A number of bacteria have been reported as entomopathogenic, and formulation of *Bacillus thuringiensis* is registered as a biopesticide in Sri Lanka since the 1990s. However, their potential has not been fully exploited by Sri Lankan farmers due to high cost and rapid breakdown of these bacteria under conditions.

13.5.2 Fungi

Mortality of insects due to fungal attack is considerably reported in the natural conditions but did not utilized one of them as a biopesticide in commercial level up to now. Recently, the Tea Research Institute of Sri Lanka focusing research on utilization of locally isolated *Beauveria bassiana* against shot hole borer, and it shows promising results under laboratory conditions (Pavithrani et al. 2009). *Metarhizium anisopliae* has a potential use to manage coconut black beetle, *Oryctes rhinoceros* (Fernando et al 1995), and the Coconut Research Institute of Sri Lanka has recommended it to manage this beetle in local conditions. *Metarhizium anisopliae* and entomopathogenic nematodes (Nematoda: Heterorhabditidae) were bioassayed against Kalotermitidae termites of tea by the Tea Research Institute of Sri Lanka, and promising strains are identified and further experiments are continuing yet.

A fungus *Trichoderma viride* normally colonizes near the rhizosphere and parasitize on pathogenic fungi such as *Phythium*, *Rhizoctonia*, and *Fusarium*. Commercial formulations of *T. viride* are available in the local market, and it provides resistance to rot and wilt diseases of many crops.

13.5.3 Insect Viruses

Insect viruses have long been considered as advantageous agents for management of insect pests of agricultural important crops, and among them, baculoviruses pay a vital role in pest management in the world. The scientist in the Coconut Research Institute at Sri Lanka did several attempts to use baculoviruses against the coconut black beetle, *Oryctes rhinoceros*, and it is recommended to use *Oryctes rhinoceros* virus (Orv) for control of coconut black beetle. Commercial formulation of *Spodoptera exigua* NPV is available in the local market and is recommended for management of caterpillars on vegetables.

13.6 Prospects of Biopesticides and Plant Disease in Sri Lanka

The economy of Sri Lanka is mainly agriculture-based. It has two sectors, namely, domestic and plantation sector. The domestic sector, which forms the dominant part of agriculture, accounts for 1.7 million farming families in a population of around 19 million. Both sectors jointly contribute 20 % to gross domestic product (GDP) and 34 % to employment (Central Bank of Sri Lanka Report 2002). In management of pests, the plantation sector approaches in a more organized manner, whereas in the domestic sector, it is more complicated due to large number of farmers, crops, and the pests involved. Pest management in Sri Lanka is mostly synthetic pesticide-dependent, and the annual imports of pesticides cost around 0.1 % of the gross domestic production in 2012. Apart from pests and weeds, plant diseases caused by various groups of plant pathogens are often a big challenge in agriculture in

Sri Lanka. Currently, protection of plants from diseases has also been largely based on the use of synthetic chemical pesticides. Applications of pesticides can have drastic negative effects on the environment, consumer, applicant, and appearance of pesticide resistance strains of the target organisms. Therefore, reduction or elimination of chemical pesticides in agriculture is highly important. One of the most desirable means to achieve this goal is by the use of new tools based on biological control agents (BCAs) and natural antimicrobial chemicals for disease control. Many studies have been devoted in Sri Lanka to the identification of microorganisms and antimicrobial botanicals that can be able to reduce activity and/or kill plant pathogens during the past two decades. When a particular country is concerned, it is important to develop its own BCAs as many quarantine procedures are imposed in almost all countries. On the other hand, BCAs are very specific in action, and many factors are involved in their mode of actions. Therefore, BCA developed for a particular plant-pathogen interaction in a country may not be suitable or not effective in another country. However, most experiences were on laboratory scale, and disease control trials were confined to experimental greenhouses or small field plots. Thus, large-scale mass production, formulations, and storage of biopesticides must be initiated, and research leading for these aspects should be given more priorities.

Up to now, BCAs against several important soilborne pathogens, foliar pathogens, and postharvest pathogens in different crops have been tested in different laboratories in Sri Lanka. For soilborne pathogens, *Trichoderma*, *Bacillus*, and *Pseudomonas* spp. have been identified for *Rhizoctonia*, *Sclerotium*, and *Fusarium* spp. on rice, chili, bean, cucumber, and banana. Soe and Costa (2012) reported that *Bacillus megaterium*, *Bacillus subtilis*, and *Aspergillus niger* isolated from the rice sheath were antagonistic to *Rhizoctonia solani*, the causal agent of sheath blight disease of rice. The talc-based formulations of these antagonists were effective as biopesticides on sheath blight pathogen. Abeysinghe (2009a) showed that *Bacillus subtilis* was effective on *Sclerotium rolfsii* on chili *and Pseudomonas* spp. introduced to the root system of bean was able to induced systemic resistance to bean rust caused by *Uromyces appendiculatus* has also reported (Abeysinghe 2009b). *Trichoderma harzianum* has also been identified as a biocontrol agent against bean rust, and induced systemic resistance (ISR) was identified as a possible mode of action (Abeysinghe 2009c). In the case of postharvest diseases Wijesinghe et al. (2011) has shown that *Trichoderma asperellum* was effective against black rot pathogen, *Thielaviopsis paradoxa* on pineapple. Jayasuriya and Thennakoon (2007) reported that one of the important soilborne fungal pathogens of rubber, *Rigidoporus microporus*, could be controlled by *Trichoderma* spp. Sivakumar et al. (2000) reported that *Trichoderma harzianum* was effective on *Nephelium lappaceum* against *Botryodiplodia*, *Colletotrichum*, *and Gliocephalotrichum*. Adikaram et al. (2002) reported that *Aureobasidium pullulans* is effective against *Botrytis cinerea* of strawberry fruits, and ISR has been identified as the mode of action. Gunasinghe et al. (2009) reported that *Flavobacterium* spp. and *Pantoea agglomerans* have been identified for *controlling Colletotrichum musae* and *Lasiodiplodia theobromae,* the postharvest pathogens of banana.

Apart from these microorganisms, some botanical extracts have also been tested against nematodes and fungal pathogens. Root nematodes in tomato have been successfully controlled by leaf extract of *Piper betle* (Premachandra et al. 2014). Field trials conducted by using *Trichoderma* bioformulation against Panama wilt pathogen *Fusarium oxysporum* f.sp. *cubense* have been successful in initial trials, and further testing is in progress (Abeysinghe et al. unpublished). Moreover, biofilm formation is one of the important aspects of root-colonizing bacteria, and efficacy of such biofilms formation is known as an important factor for success of biocontrol agents (Seneviratne et al. 2008). Therefore, numbers of research topics are currently being focused into these aspects in Sri Lanka.

As mentioned above, biological control research toward screening, formulation, and testing them under greenhouse as well as in field conditions are being conducted. However, very limited biocontrol agents are evaluated to make a successful transition from the laboratory to field. A good formulation is the key to the commercial success of biocontrol agents. Understanding the biocontrol agent can lead to innovative ways of improving the efficacy of the biocontrol product because many physical and biological parameters could be influenced to the efficacy of the product.

However, more efficient and effective ways of growing and formulating BCAs are needed in many cases in order to make biocontrol economically viable. Research on safety and environmental fate of BCAs is lacking at present. Therefore, regulatory criteria that are essential for safety have to be worked out. Additional funding is needed for biocontrol research to be progressed in Sri Lanka. In this context, importance of private/public partnerships with academia should be highlighted.

13.7 Policy Issues

The Sri Lanka National Agricultural Policy clearly mentions the importance of promoting production and utilization of organic and biofertilizer and gradually reduces the use of chemical fertilizers through integrated plant nutrition system.

In 2002, the National Engineering Research and Development Center (NERD) established the "Biogas and Biofertilizer Project at Muthurajawela in Sri Lanka," and this project was due to misunderstanding between the NERD and local authorities.

Sarvodaya Economic Enterprise Development Service (Guarantee) Limited has established effective microorganisms unit in 1996, and presently, this is the only one producer with sole agent right to produce and market effective microorganism products in Sri Lanka. They marketed several EM products for crop production.

The search of new innovative crop management programs to reduce the health risk and environmental pollution should have focused in the future research and biofertilizer, and biological pesticides will ensure these criteria in food safety and sustainability of agriculture in Sri Lanka. But there are several technological and policy gaps to promote biofertilizer and biopesticide in Sri Lanka. The major problems are lack of funds for research and fewer facilities in research laboratories with

no novel equipment for research. It is necessary to train the research scientists with no novel equipment for research, and also, it is necessary to develop international linkages of local scientist with other scientists worldwide to easily exchange their knowledge. The Sri Lanka National Agricultural Policy clearly mentions the importance of promoting production and utilization of organic and biofertilizer and gradually reduces the use of chemical fertilizers through integrated plant nutation system. But fertilizer subsidy is one of the major benefits enjoyed by farmers especially small holders and rice farmers. Rice is the staple food crop grown in Sri Lanka and very difficult to cut down the fertilizer subsidy without proper low-cost alternatives because of the political influence of the country. Fertilizer subsidy is one of the barriers to promote biofertilizer, and meanwhile more research is required to develop low-cost technology for utilization of biofertilizer as an alternative to inorganic fertilizer. More field of research on biofertilizer is still in its infancy; therefore, both laboratory and field experiments are required to fully explore potential use of biofertilizer for crop production in the future.

13.8 Current Status

Sri Lanka's agriculture have been practicing organic farming over centuries and use of biofertilizer and biopesticides has been a traditional practice followed by many rural farmers to increase the soil fertility, soil aggregate stability, water infiltration, and soil water-holding capacity. Commercial formulation of biochemical pest control agents such as semiochemicals, hormones like ecdysteroids and juvenile hormones, natural plant regulators, and enzymes is available in the pesticide market of Sri Lanka today. As well, effective microorganisms such as bacteria, fungi, viruses, nematodes, or genetically modified microorganisms effective as pest and pathogen management agents are also available in the pesticide market.

13.9 Commercialization of Biocontrol

Some of the important strategies for commercialization of biopesticides are to strengthen the commercial microbial pesticide and natural enemy industry; promote research and education on the use of biocontrol agents; exploit the export market; develop quality certification programs for biocontrol agents, requirements for commercialization research teams vs. individual isolated studies, and financial support for R&D Practical technology; improve rearing and release methods and field tests to determine efficacy, quality control, effectivity against pest, high benefit/cost, and safety for the environment. Customer services such as companies to provide detailed information on how to use their products and companies to deliver excellent customer service for site-specific biological control need to be developed. The marketplace ultimately determines the usefulness of commercial natural enemies and the viability of the industry.

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Part IV

Biopesticide and Biofertilizer Regulatory Requirements in North Asia

Commercialization and Regulatory Requirements of Biopesticides in China

14

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Abstract

The development of modern agriculture systems, based on the scientific management of water and fertilizers and excellent crop varieties, has played an important role in ensuring supplies of agricultural products and increased farmers' income in China. However, the continuous cropping of single crops and intense management of water and fertilizer has actually promoted the prevalence of plant diseases and insect pests, resulting in excess application of chemical pesticides. Currently, the problem of pesticide residue pollution is becoming a critical threat to food security and environmental health in China. To reduce the application of chemical pesticides and fertilizers, "the zero-growth plans to 2020 for the application of chemical pesticides and fertilizers" were proposed by the Chinese Ministry of Agriculture on March 18, 2015. As partial substitutes for chemical pesticide, biopesticides are receiving increasing attention from the government and the public in China. In this chapter, the current status of biopesticides in China is introduced, including an overview of the progress in developing biopesticides, the current status of the biopesticide industry, the registration status of biopesticide products, the application status of biopesticides, current registration management of biopesticides, future innovations in the registration management

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of biopesticides, important progress in research or industrialization of biopesticides, and the use of financial subsidies to promote the application of biopesticides.

Keywords
Biopesticides • Registration • Chemical pesticides • Fertilizer

14.1 Overview of the Progress in Developing Biopesticides in China

As the largest developing country, China has a population of about 1.37 billion and a cultivated land area of about 135 million hectares (National data 2014). The development of modern agriculture systems, based on the scientific management of water, fertilizers, and excellent crop varieties, has played an important role in ensuring supplies of agricultural products and increased farmers' income in China. However, the long-term, continuous cropping of single crop and the intense management of water and fertilizers have promoted the prevalence of plant diseases and insect pests, resulting in a huge demand for chemical pesticides. In 2015, the amount of chemical pesticides applied in China reached 320,000 t (The ministry of agriculture 2015). Currently, the problem of pesticide residue pollution is becoming a critical threat for the food security and environmental health in China. To reduce the application of chemical pesticides and fertilizers, "the zero-growth plans to 2020 for the application of chemical pesticides and fertilizers" were proposed by the Chinese Ministry of Agriculture on March 18, 2015 (Start-up chemical fertilizers zero growth plan 2015). As partial substitutes for chemical pesticides, biopesticides are receiving increasing attention from the government and the public, thereby increasing the opportunity to develop and commercialize biopesticides in China.

At present, there is no clear definition of a biopesticide in China. In "pesticide registration and management terms" (NY/T1667.1_l667.8_2008), a biopesticide is defined as a pesticide that controls plant pathogens, insect pests, mice, and weeds that uses directly a living organism or bioactive substances extracted from the metabolic processes of an organism, as well as synthetic substances that have the same structure compared with the natural compounds. According to their composition and sources, biopesticides are divided into six categories: microbial pesticides, botanical pesticides, agro-antibiotics, natural enemy organism, biochemical pesticides, and transgenic organisms (Ji and Wang 2010).

The study and development of biological pesticides in China began almost 70 years ago and can be divided into three stages.

In 1959, the insecticide *Bacillus thuringiensis* (Bt) was introduced in China from the former Soviet Union, which marked the beginning of the modern Chinese pesticide industry. Since then, Chinese biopesticide factories have developed several types of biopesticide products including Bt insecticide, jinggangmycin, gongzhulingmycin, and povamycin M. Notably, as the first agro-antibiotic with completely

independent intellectual property rights, jinggangmycin still works well on a range of phytopathogens, such as *Rhizoctonia solani* Kühn, *Ustilaginoidea oryzae*, and *Exserohilum turcicum*. In 1970, the Chinese State Council announced that the relevant departments were required to promote microbial pesticide research actively, whereupon the first rapid development period of biopesticide was initiated.

In 1984, the registration and management of pesticides was recovered after being abolished for decades. At that time, the regulation of biopesticides followed that of pesticides; therefore, some biopesticides were officially or temporarily reregistered in China. The officially registered biopesticides included jinggangmycin, agricultural antibiotic 120, streptomycin, gongzhulingmycin, povamycin M, gibberemycin, and Bt. Temporarily registered biopesticides included avermectin, polynactin, HaSNPV, matrine, and azadirachtin. At the same time, the Chinese government encouraged the study of biopesticides. Many projects concerning the research and development of biological control agents were listed in the National Key Technologies R&D Program of China during the seventh and eighth five-year plan period, which greatly promoted the development of the Chinese biopesticide industry. For instance, the annual yield of Bt increased from 3500 t in 1991 to 30,000 t in 1994.

Since 1994, a series of measures were proposed by the Chinese government to improve the existing biopesticide industry. Research and development on biopesticides and environmental protection were included in China's Agenda 21. The Ministry of Agriculture set up the China Green Food Development Center (CGFDC) to standardize the development of green agriculture. The application standard of biopesticides in green food was formulated. Research on biopesticides was scheduled into the National High-Technology Research and Development Program of China and the National Key Technologies R&D Program of China during the ninth five-year plan period. Since then, biopesticides have undergone rapid and healthy development, which resulted in the significant elevation of research, production, and application of biopesticides. By the end of 2001, more than 40 research institutions and 200 production enterprises had been set up. Total registered varieties of active ingredients of biopesticide accumulated to 80, which accounted for 13.9 % of China's registered varieties of active ingredients of pesticides. The number of registered products reached 696, and the total value of biopesticides was about US$ 250 million. Overall levels of agricultural antibiotics and Bt had reached international advanced levels, with some products being exported to Southeast Asia countries and the United States (Xie et al. 1999; Huang 2002; Wu and Gao 2010).

14.2 The Current Status of the Biopesticide Industry in China

By 2014, there were more than 260 biopesticide production enterprises in China, which accounted for about 10 % of pesticide production enterprises. The total output of biopesticide production was nearly 130,000 t, and its output value was about US$ 440 million, which accounted for about 9 % of the output and output value of pesticide production (Fig. 14.1) (China biological pesticide market 2014). Today,

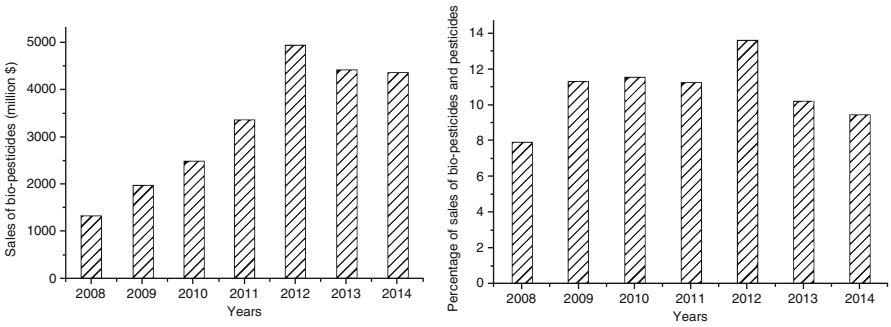

Fig. 14.1 Sales situation of biopesticide industry in China

the product level of the Chinese biopesticide industry has achieved significant progress because of continuous research efforts on biopesticides. For example, the formulation of biopesticides is more stable and diverse, and some products show slow release and highly efficient effects. On the whole, the Chinese biopesticide industry has already mastered the key technology for product development at a technical level, even reaching leading international levels in some areas, such as the artificial breeding technology of *Trichogramma* predatory mites and biocontrol agents for phytopathogenic nematodes (Qiu 2015).

14.3 The Registration Status of Biopesticide Products in China

The Institute for the Control of Agrochemicals of the Ministry of Agriculture (ICAMA) is responsible for the registration and regulation of pesticides (currently, the registration management of biopesticides refers to that of pesticides). The detailed responsibilities of ICAMA for pesticide management include registration management, product quality inspection, biological testing of product efficacy, residue testing of products, market supervision, information publication, and international cooperation. According to the Chinese Data Requirement on Pesticide Registration, biopesticides can be divided into six categories, including microbial pesticides, botanical pesticides, biochemical pesticides, natural enemies, agroantibiotics, and genetically modified organisms. Until December 2014, the number of registered types of active ingredients and total products of biopesticides were 117 and 2876, respectively (Table 14.1).

14.4 Application Status of Biopesticides in China

With farmers' increasing demands for safe pest control products and the encouragement of policies related to the application of biopesticides, in general, the overall application area of biopesticides has shown a steadily increased trend (about 10 %)

Table 14.1 Registration of products and active ingredients of biological pesticides in China (as of December 2014)

S. no.	Category	Kinds of active ingredients	Total products	Active ingredients of commodities
01	Microbial pesticides	30	281	*Bacillus* (Bt and other species of *Bacillus*), pseudomonad, *Empedobacter brevis*, *Agrobacterium radiobacter*, *Beauveria*, *Metarhizium*, *Trichoderma*, *Paecilomyces lilacinus*, *Conidiobolus thromboides*, *Aureobasidium pullulans*, *Pythium oligandrum*, *Verticillium chlamydosporium*, nuclear polyhedrosis virus, cytoplasmic polyhedrosis virus, granulosis virus
02	Botanical pesticides	29	157	Eucalyptol, star anise oil, pyrethrins, pyrethrin (I + II), physcion, nucleotide, baicalin, santonin, matrine, swainsonine, celangulin, capsaicin, chamaejasmine, triptolide, vertrine, toosendanin, hyoscyamine, cnidiadin, carvacrol, berberine, nicotine, allicin, azadirachtin, d-camphor, rotenone, camphor, methyl eugenol, sanguinarine, soybean lecithin
03	Biochemical pesticides	26	231	Oligosaccharins, brassinolide, 6-benzylaminol-purine, harpin protein, gibberellic acid, trimedlure, heteroauxin, fatty acid mixed, ethephon, plant activator protein, chitosan, muscalure, torula yeast, triacontanol, oxyenadenine, fungus proteoglycan
04	Natural enemies	5	7	*Amblyseius cucumeris*, *Microsporidia*, *Harmonia axyridis*, *Trichogramma*, *Anastatus* sp.
05	Agro-antibiotics	27	2200	Jinggangmycin, gongzhulingmycin, povamycin, kasugamycin, agro-antibiotic 120, wuyimycin, zhongshengmycin, ningnanmycin, abamectin, ivermectin, streptomycin, liuyangmycin, spinosad, cycloheximide, blasticidin S, tetramycin, nikkomycin, phenazino-1-carboxylic acid
06	Genetically modified organisms	0	0	
Total		117	2876	

(Communication from Lin Rong-hua and Yang Jun)

Table 14.2 Detailed application areas of all types of biopesticide categories

Category		Total area of the application (ha)	
		2010 (year)	2011 (year)
Natural enemies	Predatory mites	15977.33	50448
	Trichogramma	1915180	3546146
	Encarsia formosa Gahan	4217.33	6532.67
Microbial pesticides	Bt	1980113.33	2309683.33
	Bacillus cereus	6067333.33	847220
	Bacillus subtilis	13846.67	187466.67
	Trichoderma	266.67	800
	Pseudomonas fluorescens	–	533.33
	White muscardine fungus	94066.67	529633.33
	Green muscardine fungus	46000	69333.33
	Nuclear polyhedrosis virus	–	60880
	Pythium oligandrum	–	973.33
Botanical pesticides	Matrine	111180	160726.67
	Azadirachtin	82613.33	9335.33
	Rotenone	1986.67	10486.67
	Pyrethrins	–	6066.67
	Nicotine	53266.67	–
	Catechin	1200	573.33
	Berberine	–	3466.67
	Celangulin	–	20400
	Physcion	–	80
Biochemical pesticides	Ethephon	353066.67	156866.67
	Gibberellic acid	688953.33	709880
	Oxyenadenine	–	1400
	Brassinolide	145603.33	221533.33
	Oligosaccharins	–	33400
Agro-antibiotics	Abamectin	3791686.67	4195358
	Jinggangmycin	15373273.33	10596926.67
	Kasugamycin	75333.33	635666.67
	Povamycin	171093.33	182946.67
	Spinosad	2200	122593.33
	Streptomycin	272266.67	149713.33
	Ningnanmycin	139466.67	22946.66
	Wuyimycin	2373.33	1390

Source: This table is modified from Yang et al. (2014)

in the last decades in China. It is notable that the application area of biopesticides in 2012 had increased by about 40 % compared to that in 2011, because of the promotion policies of the Chinese government. At present, the total area of the application of biopesticides in China is about 26 m h annually (Yang et al. 2014). The detailed application areas of all types of biopesticide categories in 2010 and 2011 are listed in Table 14.2.

14.5 Current Registration Management of Biopesticides in China

At present, there are 12 units of laws, rules, and regulations related to biopesticide registration and management in China, including the Code of Primary Products Quality Security, Administrative Permit Law, Standardization Law, Trademark Law, Patent Law, Law on Protection of the Rights and Interests of Consumers, Agricultural Chemicals Administrative Protection Regulations, Dangerous Chemicals Management Regulations, Regulations on the Control of Pesticides, Measures for Implementation of Pesticide Management Regulations, and Data Requirement on Pesticide Registration.

In China the registration and management of biopesticides adhere to the following four principles:

(a) Scientific Principle: Following the development dynamics of biopesticides in China and abroad, the registration and management should be scientifically based.
(b) Principle of Guidance and Encouragement: According to the status of the development of the biopesticide industry in China, the industry's development should be guided by registration policies.
(c) Principle of Transparency: The opinions or suggestions of all levels of society should be heeded, including the administrative organization, research and development institutes, producers, and users.
(d) Principle of Classification Management: Different management policies should be applied to different categories of biopesticides.

According to the Chinese Data Requirement on Pesticide Registration, the required information for the registration of a pesticide product includes the address of manufacturer, chemical and physical properties, toxicology data, data on environmental influence, and information of registration abroad. So far, no special rules have been formulated for the safety evaluation of biopesticides. The entire registration procedure comprises three necessary steps (field testing, temporal registration, and formal registration) (Wang et al. 2013; communication from Lin Huarong). The following tables (Tables 14.3, 14.4, 14.5, 14.6, 14.7, 14.8, 14.9, 14.10, 14.11, 14.12) summarize the detailed requirements for different categories of pesticides (Tables 14.3, 14.4, 14.5, 14.6, 14.7, 14.8, 14.9, 14.10, 14.11, and 14.12 were modified from Lin Huarong).

14.6 Innovations in the Registration Management of Biopesticides in China

At present, there are some issues with the registration and management of biopesticides in China. The main problems include (a) lack of detection techniques and qualified testing units for the quality of biopesticide products, (b) the definition of

Table 14.3 Data requirements of new product registration of regular chemical pesticide (technical material)

S. no.	Categories	Requirements
01	Physical and chemical properties	Active ingredient concentrations, name and content of major impurities, physical and chemical parameters of products (appearance, melting point, boiling point, density or bulk density, specific optical rotation, etc.), analytical methods for active ingredients
02	Toxicological information	Acute oral, dermal and inhalation toxicity; irritation test of skin and eyes, skin sensitization test
03	Efficacy data	Efficacy test reports of the plot experiment of products in multiple regions for 2 years or more
04	Environmental test data	Acute toxicity test of birds, fish, water flea, algae, bees (oral and contact), and silkworms. If it is highly toxic or virulent to these organisms, the environmental impact experiment report must be provided
05	Residual test data	Reports of residual tests for 2 years or more

Table 14.4 Data requirements of new product registration of regular chemical pesticide (formulation)

S. no.	Categories	Requirements
01	Physical and chemical properties	Formulation, content of active ingredients, the specific name and concentration of other ingredients, physical and chemical parameters of the product, items and index of quality control, classification (according to purpose), analytical methods of active ingredients
02	Toxicological information	Tests of acute oral, dermal, and inhalation toxicity, toxic symptoms, and methods of emergency treatment
03	Efficacy data	Efficacy test reports of the plot experiment of products in multiple regions for 2 years or more (influence on the quality of harvested items, resistance research, and shelter setting)
04	Environmental test data	Acute toxicity test report of bees, birds, fishes, and silkworms (in some cases it could be relieved or exempted); if the formulation is released slowly, test reports of soil degradation and adsorption are required
05	Residual test data	Reports of residual tests for 2 years or more

biochemical pesticides which is not clear, (c) lack of scientific and systematic product quality standards (strain identification and analysis methods of microorganism, active ingredient analysis methods, quality control and storage of products), (d) the registration of technical materials and technical concentrates, and (e) relieving or exempting the required registration data on a scientific and reasonable basis. Therefore, efforts should be made to improve the management of biopesticide registration in the future, for instance, augmenting the qualified biopesticide testing

Table 14.5 Data requirements of new product registration of microbial pesticides (technical concentrate)

S. no.	Categories	Requirements
01	Physical, chemical, and biological properties	The data form of microbial pesticides is different from that of chemical pesticides and the specified requirements including analytical methods, storage stability, contamination by microbes, taxonomic status, strain authentication report, and strain code
02	Toxicological information	Proof materials that the active ingredient does not belong to known pathogens of humans or other mammals must be provided; acute oral, dermal, and inhalation toxicity tests; irritation and infectivity test of eyes; sensitization; pathogenicity (oral, inhaling, injection); other toxicological information (if necessary)
03	Environmental test data	Acute toxicity test of birds, fish, water flea, algae, bees (oral and contact), and silkworms; if it is highly toxic or virulent to these organisms, the experimental report of environmental multiplication ability must be provided
04	Other required data	The same as that of chemical pesticides

Table 14.6 Data requirements of new product registration of microbial pesticides (formulation)

S. no.	Categories	Requirements
01	Physical, chemical, and biological properties	The data form of microbial pesticides is different from that of chemical pesticides, and the specified requirements include analytical methods and storage stability
02	Efficacy data	Toxicological information
03	Toxicological information	Proof materials that the active ingredients do not belong to known pathogen of human or other mammals should be provided; additionally, six tests, including acute oral, dermal, and inhalation toxicity, irritation test of the eyes and skin, and sensitization of skins, should be provided
04	Environmental test data	The acute toxicity test report about bees, birds, fishes, and silkworms (in some cases it could be relieved or exempted)
05	Residual test data	Could apply for relief or exemption depending on the decision of temporary committee of registration

units, improving the techniques for product quality control, and eventually establishing a profound evaluation, registration, and management system of biopesticides.

The future direction of the development of biopesticide registration and management should apply to different product categories: (a) according to the properties of every category of biopesticides, specific management system should be developed for them, and (b) as for the management of agro-antibiotic pesticides, they should be carefully treated based on their properties. For those antibiotics that are accepted worldwide, the registration requirements should be simplified; however, for those

Table 14.7 Data requirements of new product registration of botanical pesticides (technical concentrate)

S. no.	Categories	Requirements
01	Physical, chemical, and biological properties	The same as that of chemical pesticides. However, if the analysis report of total fractions could not be provided for specific reasons, the proof issued from a testing agency authorized by the Ministry of Agriculture should be provided, and the one or more active ingredients must be identified
02	Toxicological information	The required information is the same as that of chemical pesticides. However, if it has been registered as a food additive, pharmaceutical, or healthcare product, the information about reproduction, teratogenesis, chronic effect, and carcinogenicity could be relieved or exempted by application
03	Environmental test data	Environmental behavior (soil degradation test, hydrolysis test, photolysis test in water), environmental toxicology (acute toxicity test of birds, fish, water flea, algae, bee, and silkworm)
04	Other required data	The same as chemical pesticides

Table 14.8 Data requirements of new product registration of botanical pesticides (formulation)

S. no.	Categories	Requirements
01	Physical and chemical properties	The same as that of chemical pesticides
02	Efficacy data	The same as that of chemical pesticides
03	Toxicological information	Six test items, including acute oral, dermal, and inhalation toxicity, irritation test of the eyes and skin, and sensitization of the skin, should be conducted
04	Environmental test data	Acute toxicity test of birds, fish, water flea, algae, bee, and silkworm; if the formulation is released slowly, test reports of soil degradation and adsorption are required
05	Residual test data	Report of residual test in multiple plots for 2 years or applying for the decision of the registration committee

antibiotics that could affect humans and animals, the registration requirements should be strengthened to prevent their injudicious use.

To promote the development of biopesticide industry, the registration and management policies of biopesticides should be adjusted to better match China's national conditions. The revised data requirements for biopesticide registration should follow the principles of shortened registration process and the simplified data requirements to streamline the registration policy. For example, the original registration process (field trials-temporary registration phase-officially registered phase) should be adjusted to field trials followed by the officially registered phase. The regulation of pesticide registration data requirement should incorporate some noteworthy changes including the following: (a) agro-antibiotics should be

Table 14.9 Data requirement of new product registration of biochemical pesticides (technical concentrate)

S. no.	Categories	Requirements
01	Physical, chemical, and biological properties	The same as that of chemical pesticides. Could apply for relief or exemption of the data of residue and environmental behavior tests if products are derived from biological fermentation and have been safely applied in agro-production for years
02	Toxicological information	Six tests, including acute oral, dermal, and inhalation toxicity, irritation test of the eyes and skin, and sensitization of the skin, are required
03	Environmental test data	Acute toxicity tests of fish, water flea, algae, bee (oral and contact), and silkworm; unless it is highly toxic, data of environmental behavior could be exempted
04	Other required data	The same as that of chemical pesticides

Table 14.10 Data requirements of new product registration of biochemical pesticides (formulation)

S. no.	Categories	Requirements
01	Physical and chemical properties	The same as that of chemical pesticides
02	Efficacy data	The same as that of chemical pesticides
03	Toxicological information	Six test items, including acute oral, dermal, and inhalation toxicity, irritation tests of the eyes and skin, and sensitization of the skin, are required
04	Environmental test data	The acute toxicity test report of silkworm (exempted categories of biochemical pesticides include insect pheromones, hormones, and enzymes)
05	Residual test data	Could apply for relief or exemption depending on the decision of temporary committee of registration

Table 14.11 Data requirement of new product registration of natural enemies (formulation)

S. no.	Categories	Requirements
01	Quality of products	Reports concerning biological characteristics, index and test methods of quality control, and verifying report of test methods
02	Efficacy data	Efficacy test reports of the plot experiments of products in multiple regions for 2 years (control objects, scope of application, control efficacy, economical efficiency)
03	Toxicological information	Information concerning bioactivity and biosecurity, influence on crops
04	Environmental test data	Influence on national protected species, beneficial organisms, untargeted organisms; possibility of hybridization with indigenous species or races and its influence

Table 14.12 Data requirement of new product registration of genetically modified organisms

S. no.	Categories	Requirements
01	Physical and chemical properties	Category of the genetically modified organism (plant, animal, or others), recipient organism, target genes, vectors, types of genetic manipulation
02	Toxicological information	Acute oral, dermal, and inhalation toxicity to mammals (rat), sensitization of the skin, safety of agricultural products
03	Efficacy data	Efficacy test reports of the plot experiment of products in multiple regions for 2 years or more
04	Environmental test data	The influence of the residual body of the genetically modified organism on the environment, including the influence of gene flow to the ecosystem, the genetic makeup, and the stability of the gene; decomposition property; influence on environmental organisms (soil microbes, birds, bees, and aquatic organisms)
05	Residual test data	Residue examination of toxicants in agro-products

Source: Tables 14.3, 14.4, 14.5, 14.6, 14.7, 14.8, 14.9, 14.10, 14.11, and 14.12 are modified from Lin Huarong

classified as a special pesticide category, and required registration materials should be adjusted according to the product features; (b) on the basis of guaranteed quality and safety of agricultural products, the area of field plot trials should be increased to shorten the time of the trials during the process of biopesticide registration (communication from Lin Huarong).

14.7 Progress in Research and Industrialization of Biopesticides in China

In the last decade, more than 20 major, national scientific projects had been deployed by the Chinese government to accelerate the research and industrialization of biopesticides. As a result, the Chinese biopesticide industry has achieved significant progress in the fields of key technology and product development. The present technological level of biopesticide products, such as Bt, agro-antibiotics, cotton bollworm nuclear polyhedrosis virus (NPV), and fungal insecticides, is very advanced. Several new activator proteins originating from microbes have been identified, and related products have been developed, which laid a foundation for the commercialization of such biopesticides. *Nosema locustae* and several new types of agro-antibiotic products have been developed and applied successfully (Qiu 2013). The "gas dual-dynamic solid-state fermentation" technique and apparatus have been developed, which overcome the shortcomings of traditional solid-state fermentation process, such as the high rate of contamination and uncontrollable fermentation parameters (Chen and Qiu 2010). Meanwhile, certain new botanical pesticides and natural enemies have been identified and developed.

14.7.1 Microbial Pesticides

According to the action targets, microbial pesticides could be divided into four types: microbiocides, microbial insecticides, microbial herbicides, and microbial growth regulators. At present, more than 100 different bacteria have been screened as insecticides in China. Among them four *Bacillus* species, *B. thuringiensis*, *B. popilliae*, *B. sphaericus*, and *B. lentimorbus*, were developed as products and have been applied practically. Recently, scientific interest in screening synergistic factors, fermentation technology, and the genetic recombination of broad-spectrum Bt is in focus. The control efficiency of the pathogenicity-enhanced Bt strain CAB109 against beet armyworm reached 75.3 %, which exceeds the control efficiency of commonly used chemical pesticides and microbial insecticides. Two hundred twenty virus strains have been isolated from 188 species of insects, of which 110 were first reported by Chinese researchers. As a candidate microbiocide, *Bacillus* is the most extensively studied genus, and applied research using *Bacillus* reached internationally advanced levels in China. Certain excellent strains have been registered as biopesticides for disease control in various crops. *B. subtilis* strain Bs-916 was registered for the control of rice sheath blight disease with stable control efficiency in the field of 60–80 %. *B. subtilis* strain B908 was registered to control *Panax notoginseng* root rot, tobacco black shank, and rice sheath blight disease. The control efficiency in the field of both *B. subtilis* strains BL03 and XM16 against apple core mold and cotton anthracnose disease reached a remarkable 90 %. The control efficiency of *B. pumilus* strain TW2 against rice neck blast was about 80 %, which was effective for the disease control. *Fusarium* and *Godronia* were reported to be highly effective for the control of wild oats and semen cuscutae, respectively (Zhu and Yin 2012; Liu et al. 2014).

14.7.2 Botanical Pesticides

The systematic study of botanical pesticide research in China started in the 1950s. To date, more than 2000 kinds of plants have been scrutinized for agro-active compounds. Plants with insecticidal activities have been identified, such as *Sabina vulgaris* Antoine, *Cynanchum hancockianum* (Maxim.) Al. Iljinski, *Senecio palmatus*, the flower bud of lily magnolia, and *Macleaya cordata* (Willd.) R. Br. In addition, many plants with insecticide or microbiocide activities have been reported. *Spinacia oleracea* L. and *Albizia julibrissin* Durazz. effectively repressed tobacco mosaic virus (TMV). *Cephalotaxus hainanensis* and other plants were allelopathic to some kinds of weeds (Zhang et al. 2015). In the last decade, the scope for screening plant candidates has expanded, and more attention has been paid to the identification of biologically active plant substances. Zhang et al. (2015) reported that some substances extracted from plants such as cinnamon, the root of red-rooted salvia, leaves of clove, lotus, and tea seeds had the potential for industrial exploitation because of their obvious preservation effects on kiwi fruit, apple, and medlar. Meanwhile, certain essential oils were reported to have insecticidal or antimicrobial activities,

and some components of these oils were identified and verified functionally (Hu et al. 2011).

The finiteness of natural resources is a bottleneck, limiting the industrialization of botanical pesticides. Based on the totipotency of plant cells, different kinds of bioreactors have been developed. At present, the applications of bioreactors include plant cell suspension culture, adventitious root development, and hairy root culture. Using these techniques, more than 100 different kinds of plants have been cultivated artificially, including pyrethrum, *Tripterygium wilfordii*, *Tagetes patula*, ginseng, *Melia azedarach*, and *Celastrus angulatus*. In addition to the target compounds, many new compounds have been discovered during the in vitro cultivation of plant tissues. For example, the new taxane compounds named sinenxans A, B, and C were identified from callus of *Taxus chinensis*. New diterpenoid and triterpenoid compounds were separated from calli and hairy roots of *T. wilfordii*. New sesquiterpenoid-like compounds were isolated from the hairy roots of *Artemisia apiacea* (Zhang et al. 2015). These discoveries illustrate the new approach for seeking new botanical candidates of biopesticides.

14.7.3 Biochemical Pesticides

According to the pesticide registration and management terms of China, a biochemical pesticide is defined as "natural or synthetic pesticides that is non-toxic toward target organisms and may be functional with the pattern of physiological regulation, mating disturbance and induced resistance for plants." The categories of biochemical pesticides include pheromones, hormones, plant growth regulators, insect growth regulators, and elicitors (proteins or oligosaccharides). At present, the total yield of the top six kinds of biochemical pesticides (ethephon, gibberellic acid, brassinolides, chitosan, fungus proteoglycans, and oligosaccharins) is estimated at 29,000 t, which accounts for 94 % of the total yield of biochemical pesticides. New technologies and new products of biochemical pesticides are constantly emerging in China. For example, products of insect sex pheromone lures have been serialized, and the key technology of the artificial synthesis of microbial or botanical metabolites has been mastered by the Chinese biochemical pesticide industry. In the field of development and application of plant vaccines, China has achieved levels comparable to the rest of the world (Qiu 2013). In the future, biomimetic synthesis and green production technology will promote the large-scale production and application of metabolites.

14.7.4 Natural Enemies

Since the 1950s, research employing natural enemies for management of agricultural pests has been conducted in China. In the 1980s, natural enemies of insect pests of important crops were investigated systematically nationwide. According to

the statistical data, more than 1000 natural enemy species for rice pests, more than 960 natural enemy species for corn pests, and more than 840 natural enemy species for cotton pests were identified. According to the category of natural enemies, more than 900 species of parasitoid wasps of *Ichneumonidae*, more than 380 species of predaceous ladybird, more than 400 species of tachinid, more than 400 species of 200 phytoseiid mites, and more than 150 species of farmland spiders were identified.

Presently, with breakthroughs in artificial diets and propagation techniques, a group of natural enemies could be produced commercially on a large scale, including *Trichogramma* spp., *Anastatus* spp., *Chrysopa* spp., *Encarsia formosa* Gahan, *Orius sauteri* (Poppius), *Eretmocerus* sp., *Thyscus fulvus* Compore et Annecke, *Chouioia cunea* Yang, and predator mites. For example, using *C. cunea* Yang to control a worldwide quarantine object (fall webworm) represents a significant achievement in China. This successful control exploited the advantages of *C. cunea* Yang, such as high parasitic rate, strong reproductive capacity, high ratio of females, and year-round convenient alternative hosts. This showed that *C. cunea* Yang could be an excellent candidate for management of fall webworm. In the *C. cunea* Yang release area, from the second year to the fifth year, the rate of attack by fall webworm was lower than 0.1 %, and the parasitic rate of *C. cunea* Yang was more than 92 %, which indicated a sustainable effect (Yang et al. 2014).

14.7.5 Agro-antibiotics

China has become the world's largest producer of jinggangmycin and abamectin, which are among the best-selling and most applied fungicide and insecticide products in China. From the viewpoints of industrial scale and technological level, three agro-antibiotics, jinggangmycin, abamectin, and gibberellin, are the leading products. Some agro-antibiotics, such as agricultural streptomycin, agro-antibiotic 120, povamycin, and zhongshengmycin, were listed as staple and backbone products of the microbial pesticide industry in China (Ren 2014).

In the last decade, some new varieties of agro-antibiotics have been developed in China, such as wanlongmycin reported by the Institute of Plant Protection of Guangdong Province, zuelamycin reported by the Northwest Agriculture and Forestry University, antimycin reported by the Microbiology Institute of the Zhejiang Academy of Agricultural Sciences, aureonucleomycin and phoslactomycin reported by the Shanghai Pesticide Research Institute, meilingmycin reported by the Jiangxi Agricultural University, and polaramycin reported by the Chinese Academy of Medical Sciences (Qiu 2013). With the progress in bioengineering technology, some important properties, such as efficiency and safety, of agro-antibiotics were improved by remodeling the existing agro-antibiotics via biological synthesis and chemical semi-synthesis. Meanwhile, significantly improved yields of antibiotics were realized using genetic engineering (Chen et al. 2014).

14.8 Financial Subsidies: Promoting the Application of Biopesticides in Beijing City

To promote the application of biopesticides, a financial subsidy policy was put into practice by the Chinese Ministry of Agriculture in 2014. To establish a standard system of financial subsidies, pilot schemes of the first batch of financial subsidies were conducted in some counties of Beijing City, Shandong Province, and Shanghai City (Qiu 2015). The exploratory system of financial subsidies of Beijing City will be introduced in detail.

Beijing is not only the capital of China but also the economical center and one of the biggest cities. Currently, Beijing is suffering from pollution from different sources including industrial emission, vehicle exhaust, and pesticide residues. To relieve the pressure from environmental pollution, large amounts of money have been invested to seek feasible methods to counter environmental pollution. Among them, promoting the application of biopesticides by providing allowances to growers has accelerated the application of biopesticides. So far, four different approaches have been established to subsidize the use of biopesticides.

The first model is the subsidized sale model that was trialed in Renshou Town, Changping District. Customers buy the biopesticide products listed in the subsidy catalogue from agricultural materials stores at a subsidized price. Only growers who hold debit cards of agricultural capital subsidies for strawberry planting are qualified for the allowance. Biopesticide products are subsidized by 80 %, and other chemical pesticides with high efficiency, low toxicity, and low-residue properties are subsidized by 50 %. The second model is the chain delivery model, which was trialed in Tongzhou District. The chain delivery system, comprising agricultural sales agencies and local agricultural services, distributes subsidized biopesticides to growers directly. The third model is the village committee delivery system, which was trialed in Fangshan District. This system comprises the plant protection station of Fangshan District, the agricultural offices of local towns, and local village committees. Subsidized biopesticides are distributed from the plant protection station of Fangshan District to the local village committees via agricultural offices of local towns. The fourth model is the specialized service organization delivery system trialed in Daxing District. Basing on the local situation of plant diseases and insects, the subsidized biopesticides were distributed to technicians of local specialized crop cooperation services, and these technicians provide a unified service for pest control (Yue et al. 2014).

14.9 Conclusion

Through long-term efforts of the government, scientists, and industries, the Chinese biopesticide industry has made great achievements. However, there are still some obstacles to the ongoing development of the Chinese biopesticide industry. Firstly, some innate disadvantages, including relatively higher controlling cost, unstable controlling effects, and higher knowledge background requirement for users

compared with that of chemical pesticides, have meant that, in practice, many farmers consider biopesticides as the last choice for pest control. Secondly, it is very difficult to detect pesticide residues of agricultural products widely in such a large market as China, and it is impossible for consumers to distinguish harmless agricultural products from their harmful equivalents. Agricultural products subjected to improperly applied chemical pesticides for pest control may be produced at a relatively lower cost; therefore, they are more competitive in the market. Thirdly, in the pursuit of maximum profits, many big pesticide enterprises are reluctant to recommend biopesticides to the farmers. By contrast, although some small pesticide enterprises wish to develop biopesticides to survive in the fiercely competitive market, they seldom possess adequate financial and technological capacity to support the development of new biopesticide products. Biopesticides could be a powerful aid to sustainable agriculture. They are the most likely source of alternatives to some of the most problematic chemical pesticides that may severely affect the health of consumers and environment. Therefore, the prospects of biopesticides are bright, because they represent an ultimate solution to the problems including pest resistance to traditional chemical pesticides and the side effects of pesticides on the surrounding environment and on human health (Qiu 2015).

Promoting the development of the biopesticide industry to guarantee the safety of the environment and the food chain has become an important concern of the Chinese government and the public. To alleviate this concern, some measures should be taken or enhanced, such as the support of policy and finance, intensified supervision of pesticides, and decreased registration cost (time and capital). It must be noted that although it is important to encourage the application of biopesticides to aid the development of sustainable agriculture, biopesticides are not absolutely safe: some biopesticide products still have potential hazards to the environment and human health. Therefore, risk assessment of biopesticide products should be enhanced in the future to establish a rigorous risk assessment system for biopesticide products (Yang et al. 2014).

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The Registration and Regulation of Biopesticides in Taiwan

15

Tsung-Chun Lin, Tang-Kai Wang, Hua-Fang Hsu, and Ruey-Jang Chang

Abstract

In Taiwan, the biopesticides are divided into three categories including natural products, biochemical agents, and microbial agents. A total of 52 agro-pesticide permits of biopesticide have been registered in Taiwan. Most of biopesticides are classified as microbial agents, and the majority components of microbial agents are *Bacillus thuringiensis* and *Bacillus subtilis* which have 24 and 10 agro-pesticide permits, respectively. According to the statistics of agro-pesticide production report, the biopesticide sales volume in Taiwan was 0.401, 0.295, and 0.225 % of total pesticide sales volume in 2009, 2010, and 2011, respectively. The amount of sales volume was far lower compared to the global biopesticide sales volume (6.46 %). Besides, the requirements of food safety from consumers and the booming of organic farming contribute to accelerating the development of biopesticides. In dealing with the need of more biopesticides, the competent authority for agro-pesticide management, Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), Council of Agriculture (COA), has decreased the requirements of biopesticide registration on the basis of product safety; if the biopesticide is developed by using local microorganisms as active ingredients, the testing data of toxicology required for product registration has been reduced to only three items, including acute oral toxicity/pathogenicity, acute dermal toxicity/pathogenicity (conditional, depends on the formulation of biopesticides), and acute pulmonary toxicity/pathogenicity data. In addition, it does not need to provide the data of wettability, stability test, and preheat

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treatment for heat tolerance. Furthermore, BAPHIQ continued to revise two key regulations on agro-pesticide registration in 2013, such as "standards for physicochemical property tests and toxicology testing of agro-pesticides" and "standards for agro-pesticide field test," which is a step further to reduce technical barriers in achieving the goal of promoting biopesticide production. However, biopesticide registrants encounter several problems in Taiwan. First, the complex biological characteristics of microbes are difficult to fit the unique regulation of product specifications. Second, the quality control data is related to the business confidentiality. Third, the requirements of constructing biopesticide factory need to be clarified. Finally, shall biopesticides be listed as least-regulated pesticides? To resolve the abovementioned problems, it is necessary to establish a single-desk unit to provide the preregistration consultation and regulation advice for industry and to set up the standard procedures of research, invention, and registration for enhancing the development of biopesticides.

Keywords
Biopesticide • Agro-pesticide Management Act • Microbial agent • Agro-pesticide registration

15.1 Introduction

Taiwan is located on the junction of tropical and subtropical areas; the warm and humid climate and intensive multiple cropping system have facilitated the development of plant diseases and pests, which are important limiting factors to the agricultural production. With the agricultural trade liberalization and accession of Taiwan to the World Trade Organization (WTO), the imports of agricultural products to Taiwan have been increasing vastly, resulting in the high risk of invasion by exotic plant diseases and pests. In view of ensuring the yield and quality of crops, the easy and simple way for control of pests and diseases is by using synthetic pesticides.

In 1962, Rachel Carson noticed that the use of chemical pesticides was causing damage to the nontarget organisms and ecological systems (Carson 1962). Thereafter, farmers gradually realized the importance of environmental protection and initiated to reform the cultivation methods such as ecological farming, nontoxic agriculture, and organic farming. By this trend, biopesticides had been attracting researchers' attention. Biopesticides in general are considered as safer and more specific and pose less residual problem as compared to chemical pesticides. With these characteristics, the biopesticide has gained public attention because it fits the consumers' demand for food safety.

According to the "Agro-pesticide Act" Article 5 of Taiwan, the formulated agro-pesticides including the chemicals or biologically based formulations are listed below: (1) those used for preventing and eliminating pests of crops and forest or the

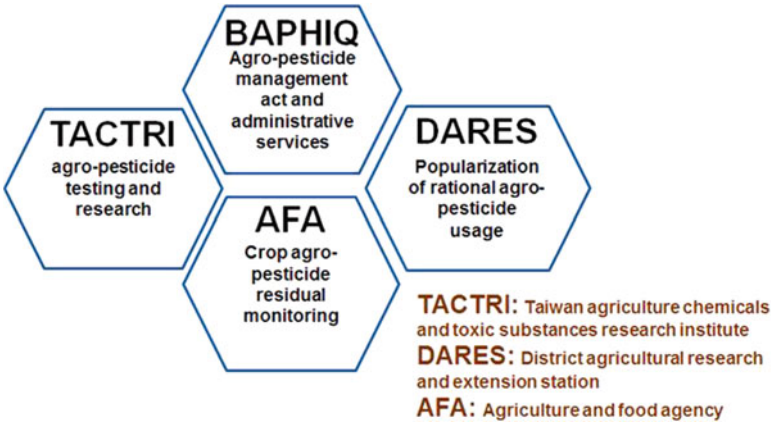
BAPHIQ
Agro-pesticide management act and administrative services
TACTRI
agro-pesticide testing and research
DARES
Popularization of rational agro-pesticide usage
AFA
Crop agro-pesticide residual monitoring
TACTRI: Taiwan agriculture chemicals and toxic substances research institute
DARES: District agricultural research and extension station
AFA: Agriculture and food agency

Fig. 15.1 Role of central competent authority for agro-pesticide management in Taiwan

products thereof, (2) those used for regulating the growth of crops and forest or for influencing the physiological functions thereof, (3) those used for regulating the growth of beneficial insects, and (4) any other chemicals designated by the central competent authority for protecting plants that belong to the scope of pesticide management by the government (Agro-pesticide Act 2007). The competent authority for pesticide management is the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ), Council of Agriculture (COA), at the central level and the local government at the regional level. Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI), COA, is in charge of pharmacology and toxicology tests of agro-pesticide. Agriculture and Food Agency (AFA), COA, monitors the pesticide residues and harmful substances in crops. The district agricultural research and extension stations are responsible for counseling the safe use of agro-pesticides to farmers (Fig. 15.1).

Biopesticides are divided into three categories including natural products, microbial agents, and biochemical agents. The natural products refer to those natural ingredients which are not chemically refined or synthesized, e.g., pyrethrum. The microbial agents are used for controlling the pests or diseases of crops, e.g., *Bacillus thuringiensis* and *Bacillus subtilis* or their active ingredients. The biochemical agents mean biological ingredients are extracted with chemical solvents or synthesized; the control methods do not directly kill pests but attract them, e.g., as pheromones (The standards for physico-chemical property tests and toxicology testing of agro-pesticides 2013).

Owing to food safety requirements of consumers, the development of biopesticides is more important and urgent with each passing day. In order to accelerate the development and registration of biopesticides in Taiwan, it is necessary to understand the "Agro-pesticide Act" and registration procedures for biopesticides.

15.2 The Biopesticide Industry in Taiwan

According to the estimation of BCC Research (USA), biopesticides accounted for approximately 6.46 % of global pesticide sales volume in 2014 (Fig. 15.2), and the compound annual growth rate of biopesticides was 15.6 % from 2009 to 2014 (BCC Research 2010). However, the biopesticide accounted for only 0.401 %, 0.295 %, and 0.225 % of pesticide sales volume in Taiwan in 2009, 2010, and 2011, respectively (Fig. 15.3); the ratio is far lower than the global market. It means the promotion of biopesticides for crop protection is not sufficient and the development of biopesticides has a great potential in Taiwan.

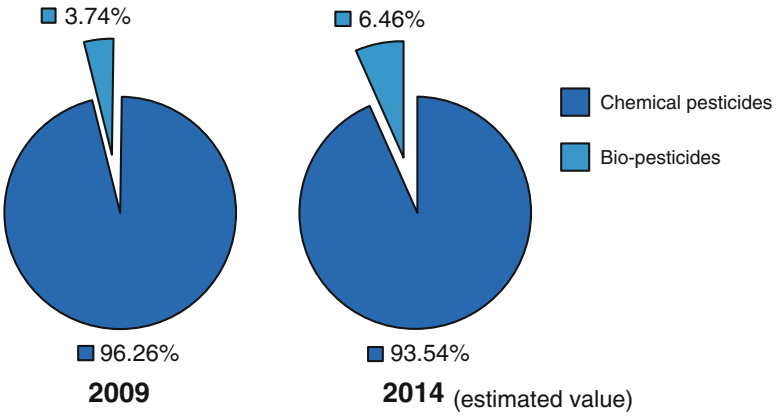

Fig. 15.2 The global sales volume of biopesticides and chemical pesticides

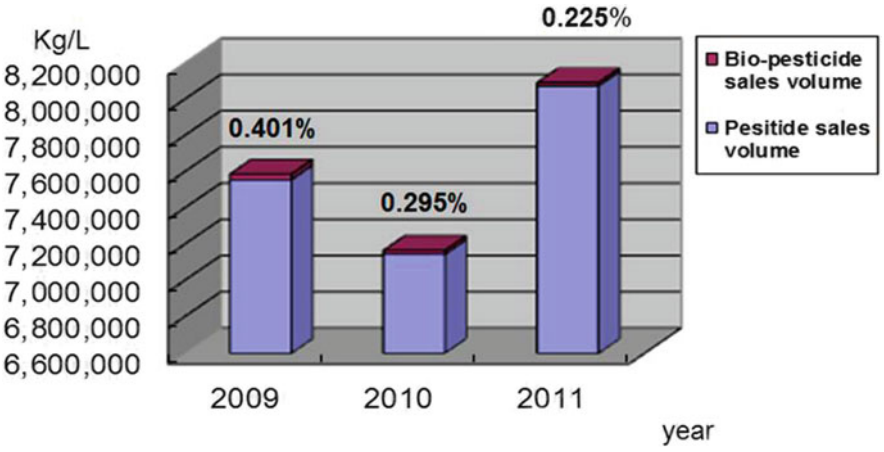

Fig. 15.3 The domestic sales volume of biopesticides and chemical pesticides in Taiwan

Table 15.1 Biopesticide registration status in Taiwan

Kinds of biopesticides		Number of licenses	Number of companies
Microbial agents			
Fungicides	*Bacillus amyloliquefaciens*	1	1
	Bacillus subtilis	10	6
	Streptomyces candidus	1	1
	Trichoderma virens strain R42	1	1
Insecticides	*Bacillus thuringiensis*	24	13
	Spodoptera exigua NPV	1	1
Biochemical agents			
Pheromones	Tobacco cutworm	3	1
	Sweet-potato weevil	2	1
Attractants	Methyl eugenol	4	4
	Cuelure	3	3
Natural products			
Insecticides	Pyrethrum	1	1
	Azadirachtin	1	1
	Rotenone	1	1
Total		53	35

Currently, the registered biopesticides in Taiwan contain *Bacillus amyloliquefaciens*, *B. subtilis*, *B. thuringiensis*, *Streptomyces candidus*, *Trichoderma virens*, pyrethrum, azadirachtin, pheromones, etc. (Table 15.1). There are 53 pesticide licenses of biopesticides including 38 microbial agents, 12 biochemical agents, and 3 natural products. Among them, *Bacillus subtilis* and *B. thuringiensis* have 10 and 24 licenses, respectively (pesticide information service network, http://pesticide.baphiq.gov.tw). In coping with the need of consumers, BAPHIQ promoted the development of biopesticide industry in Taiwan, and appealed the district agricultural research and extension stations and "the Farmers' Academy" to educate farmers regarding the use of biopesticides through various training courses. At present, BAPHIQ intends to strengthen the utilization of research results from domestic research institutes.

15.3 The Registration and Regulation of Biopesticides in Taiwan

Registration of biopesticide products shall comply with the requirements of "Agro-pesticide Act," including (I) standards for physicochemical property tests and toxicology testing of agro-pesticides, (II) standards for agro-pesticide field test, and (III) standards for biopesticide factory. All of the documents required for

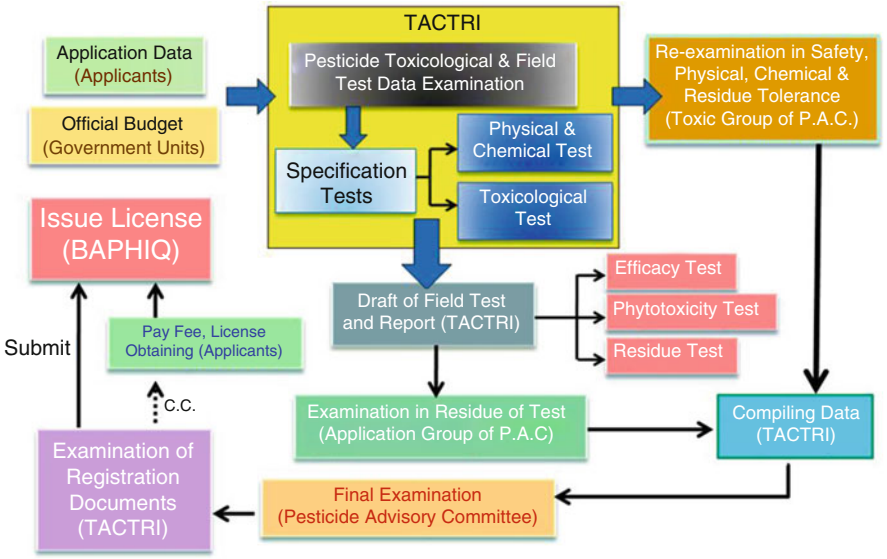
TACTRI
Application Data (Applicants)
Official Budget (Government Units)
Pesticide Toxicological & Field Test Data Examination
Specification Tests
Physical & Chemical Test
Toxicological Test
Re-examination in Safety, Physical, Chemical & Residue Tolerance (Toxic Group of P.A.C.)
Issue License (BAPHIQ)
Submit
Pay Fee, License Obtaining (Applicants)
C.C.
Draft of Field Test and Report (TACTRI)
Efficacy Test
Phytotoxicity Test
Residue Test
Examination in Residue of Test (Application Group of P.A.C)
Compiling Data (TACTRI)
Examination of Registration Documents (TACTRI)
Final Examination (Pesticide Advisory Committee)

Fig. 15.4 The application process of agro-pesticide registration

registration should be submitted to the designated single window (TACTRI) and sent to Pesticide Advisory Committee (PAC) for reviewing. After completing the processes, applicants will be issued the licenses for production and commercialization of biopesticides (Fig. 15.4, http://www.tactri.gov.tw).

15.3.1 The Standards for Physicochemical Property Tests and Toxicology Testing of Agro-pesticides

The requirement of documents for registration of biopesticides is relatively less than those of chemical pesticides. The required data of chemical and physical properties, toxicology testing items, active ingredients, and impurities of biopesticides are less than those required from chemical pesticides (Tables 15.2 and 15.3). According to the standards for physicochemical property tests and toxicology testing of agro-pesticides Article 2, the physicochemical property tests and toxicology testing of biopesticides should be conducted in accordance with the principle of good laboratory practice (GLP) and test regulations established by the central competent authority (BAPHIQ). At present, TACTRI and other 14 private companies have established the toxicology testing laboratories accredited by the Taiwan Accreditation Foundation (TAF, the only national accreditation body in Taiwan, http://www.taftw.org.tw). After the biopesticides of private companies have passed the toxicology testing, it will be more conducive for those biopesticides to expand their global markets.

Table 15.2 The revised agro-pesticide's physicochemical property test items

Chemical and physical properties	Substance to be tested		Chemical agro-pesticide	Biochemical agro-pesticide	Microbial agro-pesticide	Notes
Physical state	Technical grade agro-pesticide	Formulated agro-pesticide	○	○	○	1
Color	Technical grade agro-pesticide	Formulated agro-pesticide	○	○	○	
Odor	Technical grade agro-pesticide	Formulated agro-pesticide	○	○	○	
pH	Technical grade agro-pesticide	Formulated agro-pesticide	○	△	○	2
Melting point or boiling point	Technical grade agro-pesticide		○	○	×	3
Density, specific gravity, bulk density	Technical grade agro-pesticide	Formulated agro-pesticide	○	○	△	4
Vapor pressure	Technical grade agro-pesticide		○	○	×	5
Solubility	Technical grade agro-pesticide		○	○	×	6
Partition coefficient	Pure product		○	○	×	7
Dissociation constant	Pure product		○	○	×	8
Viscosity	Technical grade agro-pesticide	Formulated agro-pesticide	○	○	○	9
Stability	Technical grade agro-pesticide		○	△	×	10

(continued)

Table 15.2 (continued)

Chemical and physical properties	Substance to be tested		Chemical agro-pesticide	Biochemical agro-pesticide	Microbial agro-pesticide	Notes
Flammability	Technical grade agro-pesticide	Formulated agro-pesticide	○	△	×	11
Miscibility	Technical grade agro-pesticide	Formulated agro-pesticide	△	△	△	12
Explodability	Technical grade agro-pesticide	Formulated agro-pesticide	○	△	×	13
Corrosive characteristics	Technical grade agro-pesticide	Formulated agro-pesticide	○	○	○	14
Storage stability	Technical grade agro-pesticide	Formulated agro-pesticide	○	○	○	15
Others	Technical grade agro-pesticide	Formulated agro-pesticide	△	△	△	16

○ mandatory test results, △ shall be required, × results not required

Notes

1. Description of the appearance, such as solid, granules, volatile liquid, etc.

2. Meaning the relative amount of H2SO4 or NaOH or pH value. Those that shall be diluted or dissociated in water shall be tested at a temperature between 20 °C and 25 °C. If the properties are well characterized and the chemical is identical to a registered chemical structural group of pheromone (such as straight-chain lepidopteran pheromones, SCLPs), the results are not required

3. For agro-pesticides that are in liquid form at room temperature, information of the boiling point (or boiling range) is required; for agro-pesticides that are in solid form at room temperature, information of melting point (or melting range) is required

4. Provide the information for chemical agro-pesticide and biochemical agro-pesticide if the pesticide is liquid or solid at room temperature; for microbial agents, provide the information for its formulated products

5. Shall be tested at 25 °C. Not needed if the boiling point is below 30 °C

6. Solubility means in distilled water and other representative polar and nonpolar solvents at 20 °C or 25 °C

7. Octanol-water partition coefficient is required generally for nonpolar, organic substance

8. Shall be required depending on the case. If the applicant believes there is no need to provide this information, state the reasons supporting so

9. Viscosity is required if the agro-pesticide is in liquid form at room temperature

10. Stability including the (a) sensitivity to metal and light and (b) stability at room temperature and other temperatures of the agro-pesticide. If the properties are well characterized and the chemical is identical to a registered chemical structural group of pheromone (such as SCLPs), the results are not required

(continued)

Table 15.2 (continued)

11. If the product consists of inflammable liquid, provide information of flash point (open cup and closed cup); if consists of inflammable solid, provide information of ignition propensity. If the properties are well characterized and the chemical is identical to a registered chemical structural group of pheromone (such as SCLPs), the results are not required
12. Miscibility is required if the agro-pesticides are used as liquid formulation or need to be mixed and diluted in petrochemical solvents. If the properties are well characterized and the chemical is identical to a registered chemical structural group of pheromone (such as SCLPs), the results are not required
13. Explodability is required if the product contains potential explosive substance. If the properties are well characterized and the chemical is identical to a registered chemical structural group of pheromone (such as SCLPs), the results are not required
14. Corrosive characteristics to the packaging materials shall be included in the storage stability
15. Stability test is required for at least 1 year (at room temperature), or heat resistance test at 54 °C for 14 days, or other internationally recognized test method
16. If the product has unique properties, it shall be required to provide other information of the physicochemical property according to case assessment

15.3.2 Standards for Agro-pesticide Field Test

Prior to implementing the field trials, biopesticides shall confirm the specification inspection, and the design of their domestic field test shall be approved by the central competent authority (BAPHIQ). The field test of the biopesticides (including efficacy test, hazard test, and residue test) shall be implemented in more than three different locations, and at least one of them shall be conducted domestically. Although the reports of foreign field tests are acceptable in Taiwan, the efficacy of biopesticides should be confirmed again, domestically. The efficacy test is divided into the complete test and verification test. The control treatment with the similar type of registered biopesticides is required for both tests. If the candidate biopesticide is a brand-new active ingredient and the same type of product is not available for comparison, the efficacy test report with untreated group as control is acceptable. In the case of hazard test of biopesticides, it shall be carried out on at least two different varieties of the same crop.

15.3.3 Standards for Biopesticide Factory

A biopesticide manufacturer shall establish a biopesticide factory for only biopesticide production. In addition, the factory registration has to be approved in accordance with the relevant laws and regulations. The factory established shall comply with the agro-pesticide factory establishment criteria. Owing to the risk of diffusion pollution, the workplace for producing biopesticides should have sterilization equipment and independent air-conditioning systems. The biopesticide factory should hire full-time technical staffs with expertise on microorganism, agrochemistry, plant disease, plant pest, pharmacy, biochemistry, or related degrees and who have at least 2 years of practical work experience or with technical qualifications.

Table 15.3 The revised regulations of the microbial agents' toxicological testing items

Item	New active ingredient		New formulation or content	New range of use	Approved registration	Sample for testing		Notes
	Food crop	Nonfood crop				Technical grade agro-pesticide	Formulated agro-pesticide	
I. Acute toxicity/pathogenicity studies								
Acute oral toxicity/ pathogenicity	○	○	△	*	*	ˇ	ˇ	1
Acute dermal toxicity	○	○	△	*	*	ˇ	ˇ	1
Acute pulmonary toxicity/ pathogenicity	○	○	△	*	*	ˇ		
IV, IP injection	△	△	×	×	×	ˇ		2
Acute dermal irritation	△	△	×	×	×	ˇ	ˇ	3
Acute eye irritation	○	○	△	*	*	ˇ	ˇ	3
Skin sensitization	△	△	×	×	×	ˇ	ˇ	4
Cell culture	△	△	×	×	×	ˇ		5
II. Nontarget organism toxicity/pathogenicity studies								
Aquatic toxicity/pathogenicity	△	△	△	△	×	ˇ	ˇ	6
Avian toxicity/pathogenicity	○	○	×	×	×	ˇ		
Nontarget plant toxicity	△	△	×	×	×	ˇ		7
Predator/parasite toxicity	△	△	×	×	×	ˇ		8
Honey bee toxicity/ pathogenicity	△	△	△	△	△	ˇ	ˇ	9
III. Others	△	△	△	△	×			10

○ mandatory test results, × results not required, △ shall be required depending on the case, * summary
√ items required for registration of technical grade agro-pesticide or formulated agro-pesticide

Notes

1. Applicants for the registration of technical grade agro-pesticide shall provide testing on pathogenicity studies. Applicants for the registration of formulated agro-pesticide shall provide testing on acute toxicity studies (LD50)

2. Intravenous (IV) injection pathogenicity studies are applicable for bacterial and viral products; intraperitoneal (IP) injection pathogenicity studies are applicable for fungal and protozoan products

3. Acute eye and dermal irritation testing is required on an animal testing, preferable with albino rabbits. Products that have a pH value <2 or >11.5 are exempted from this testing, but are required to label on the product with warnings of eye and dermal corrosive toxicity and severe acute eye and dermal irritation. Applicants for the registration of technical grade agro-pesticide shall provide the testing results of the technical grade agro-pesticide. Applicants for the registration of formulated agro-pesticide shall merely provide the testing results of acute eye irritation

4. Required for products that contain genetically modified microorganisms or for those with frequent contact toward the skin, inhalation, and human body

5. Results of products that contain genetically modified viruses are required

6. Results of products that contain genetically modified microorganisms or are to be used in aquatic environment (including rice paddy field) are required. If other ingredients are highly toxic or have synergistic effect, the results of aquatic acute toxicity testing of the formulated agro-pesticide shall be provided

7. Results of products that contain genetically modified microorganisms or microbial herbicides are required. The plant species for testing depend on each individual case

8. Products that contain genetically modified microorganisms or microbial insecticides used in parasite release sites are required to provide the results of toxicity/pathogenicity test on the parasite

9. Unless the method of use poses low risk to pollinators (such as indoor usages), all other methods of use in the fields for nectar plants shall provide honey bee pathogenicity testing results of technical grade agro-pesticide. Other ingredients added in formulated agro-pesticide known to be toxic to honey bees are required to provide the testing results of acute contact toxicity to honey bee adults of the formulated agro-pesticide. Nectar plants include: buckwheat, citrus, longan, lychee, melons, orange, pomelo, wax apple, loquat, starfruit, guava, plum, Chinese plum, peach, pear, strawberry, tea, camellia, canola, Astragalus sinicus, Sesbania cannabina, garden cosmos, Bidens pilosa var. radiata, Bidens pilosa var. pilosa, sunflower, kumquat (fortunella), lemon, asparagus, broccoli, cabbage, cauliflower, orange jessamine, melaleuca, leucaena, and Chinese tallow tree. Pollen plants: rice, maize, sorghum, millet, wheat, barley, garden cosmos, sunflower, Bidens pilosa var. radiata, Bidens pilosa var. minor, Bidens pilosa var. pilosa, asparagus, broccoli, cabbage, cauliflower, grape, kumquat (fortunella), lemon, loquat, orange jessamine, melaleuca, leucaena, Chinese tallow tree, and nutgall tree

10. According to the provided results, further results of toxicological testing shall be required if there are safety concerns

Under the premise of ensuring products' safety, the procedures of registering biopesticides have been relevantly simplified, such as the domestic manufactured biopesticides that consist of microorganisms isolated from the natural environment in Taiwan and have not undergone artificial mutation or genetic modification; the toxicological testing items are acute oral toxicity/pathogenicity, acute dermal toxicity/pathogenicity (conditional, depends on the formulation of biopesticides), and acute pulmonary toxicity/pathogenicity studies. Owing to the differential characteristics of biopesticides from those of chemical pesticides, the chemical and physical properties such as melting point or boiling point, vapor pressure, solubility, partition coefficient, dissociation constant, stability, flammability, and explodability are not necessary for registration of biopesticides. These amendments have proved very useful for early registering of biopesticides.

15.4 Frequently Asked Questions of Biopesticide Registration in Taiwan

The researchers and inventors dealing with the registration of biopesticides lack the understanding of "Agro-pesticide Act." Thus, the documents or information required for registration are usually not ready for reviewing. For example, the active ingredients of the microbial agents will be differed with microorganisms cultured in different media, resulting in difficulties in registration. Prior to registration, the applicants can consult experts at single window of TACTRI to ensure the validity and integrity of the required documents. Basically, to confirm the active ingredients of biopesticides (including microorganisms themselves and its metabolites), the applicants should establish the procedures for identification of the active ingredients of biopesticides, and the microorganisms themselves should be preserved at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute in Taiwan for further examination.

Although biopesticides are usually concerned with characteristics of high security, low toxicity, zero residue, and low environmental hazard, the individual products have yet to assess whether or not to classify as least-regulated agro-pesticides. To avoid the side effects of "bad money drives out good" and to ensure the quality of the products, biopesticides are still registered in accordance with the regulations of the "Agro-pesticide Act."

15.5 The Future Direction of Efforts to Agro-pesticide Management

Undeniably, agro-pesticide management in Taiwan is far from perfect; however, after reviewing the assessment and consideration of the needs of agro-pesticide industry, the following five points listed by the competent authority for pesticide management (BAPHIQ) should be addressed.

15.5.1 Improvement of Agro-pesticide Regulations and the Registration System

Indeed, to build a positive business environment for agro-pesticides and industrial development, the reasonable amendments of relevant laws and regulations are urgently needed. Aiming to increase transparency and more convenient way for registration of agro-pesticides, the existing registration system should be actively reviewed. Eliminating the high-risk agro-pesticides and creating a quick registration system for low-risk biopesticides are needed to accelerate the registration, commercialization, and field application of these low-risk biopesticides.

15.5.2 Improvement of the Business Environment of Biopesticide Retailers and Increasing Their Competitiveness

Actually, for improving the business environment of agro-pesticides, retailers should increase their professional knowledge of biopesticides; in the evaluation of biopesticides, retailers need to be continuously educated, and incentives should be given for competent retailers. To improve the products' quality and reduce production costs, the plans for the mergers and acquisitions of biopesticide companies or integration of their products have to be promoted. The biopesticide industries need to expansively use the existing favorable measures of export processing zone for those imported and dedicated for processing and exporting those manufactured or processed exclusively for exporting and coordinate with the policy of "free economic pilot zones (FEPZs)" promoted by the central government of Taiwan to establish the processing center of biopesticides.

15.5.3 Promotion of Reducing the Use of Chemical Pesticides and Assessment of High-Risk Agro-pesticides

To strengthen the promotion of rationale and proper use of chemical pesticides, educating farmers by district agricultural research and extension stations and the Farmers' Academy is required urgently. For pursuing the ultimate goal of mass production, commercialization, and field application of biopesticides, the technological transfer of biopesticides (containing the information and documents required for registration) from researchers to agro-pesticide industry through production and study cooperation is needed to be actively promoted. The assessment and elimination of high-risk agro-pesticides (such as acute toxicity and environmental hormone-like pesticides) is needed to be implemented continuously, while the safety assessment of active ingredient and other components of agro-pesticides is also needed to be carried out simultaneously.

15.5.4 The Safety Management of Agricultural Products

Aiming to achieving the goal of establishing a "seamless safety management system of agricultural products," it is needed to execute the three measures to strengthen the setup and management of basic agricultural environment, strengthen the management for healthy crop production, and strengthen the safety management of agricultural products. Resolving the regulatory issues of agro-pesticides and illegal use of agro-pesticides and ensuring the health and safety of agricultural products, the measures of "extended use scope or minor use" of agro-pesticides and setting maximum residual limits (MRLs) of agro-pesticides for various crops should be carried out actively. If Taiwan try to resolve the differences of MRLs for agricultural products between Taiwan and trading countries, it is important to build the ability of negotiating the international standards for MRLs of agro-pesticides.

15.5.5 Investigating and Seizing of Illegal Agro-pesticides

The investigating and seizing of illegal agro-pesticides are needed to execute in conjunction with local governments and judicial police (if necessary). It is important to effectively monitor manufacturing, sale, and use of legal agro-pesticides and reduce the flow of illegal agro-pesticides, the control measures for the flow of legal agro-pesticides are needed to be implemented. A stringent control measure of customs and coast guard units should be adopted for strengthening the border control and preventing the inflow of illegal agro-pesticides.

15.6 Conclusion

The agricultural production, development of agriculture industry, healthy food, the security of environment, and ecological system are all closely related to the agro-pesticide management. The management of biopesticides is the responsibility of the government mainly, however the research, development, and commercialization of biopesticides are heavily relied on the supervision of researchers, industries, and farmers for achieving the goal of sustainable agriculture. It is crucial that collaboration among all stakeholders including regulatory agency, scientists, industries, farmers and consumers to ensure a successful production and commercialization of biopesticides in Taiwan.

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Part V

Biopesticide and Biofertilizer Regulatory Requirements in West Asia

Biorational, Environmentally Safe Methods for the Control of Soil Pathogens and Pests in Israel

16

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Abstract

Since 1992, 74 % of the plant protection products have been removed from the European market. While this process resulted in the withdrawal of most of the pesticides applied to the soil, alternative nontoxic solutions to these chemicals are lacking. Clearly there is an acute need for biorational control strategies for the management of soil pathogens and pests. Here we review the use of compost, biofumigation, and bacterial biological control. While these measures were first intended for sustainable and organic agriculture, we believe they will be adopted in the near future by the conventional sector. The pathogen- and pest-suppressive capability of composts is associated with microbial activity of bacterial and fungal populations in the rhizosphere and the interactions between microbials and macrobials in the soil. Biofumigation is a cultural method to treat soilborne pathogens and pests, using green manures, as well as by crop rotation, intercropping, and pure compound amendments as seed meal, dried plant material, or soil mulching. Biofumigation technology demonstrates the potential to reduce environmental pollution through the replacement of toxic synthetic pesticides with biodegradable plant secondary metabolites. While there is considerable literature on the use of biofumigation on soil pathogens and nematodes, much less can be found on the control of soilborne insect pests. Here the emphasis will be on field

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rather than laboratory experiments. We also present studies on the compatibility of biofumigation with natural enemies.

Being a soil pathogen, *Fusarium oxysporum* is difficult to control, and *Fusarium* prevention is limited. Two groups of bacterial biocontrol agents for Fusarium wilt disease and Fusarium crown disease were investigated and developed: endophytic bacteria living within the plant and plant growth-promoting rhizobacteria (PGPRs) that colonize the rhizosphere. Here, we focus on the latter. Finally we conclude by presenting results from Israel using biofumigation for pathogen and pest control and hypovirulent isolates for the control of Fusarium wilt.

Keywords
PGPR • Fusarium wilt • Biofumigation • Sustainable agriculture

16.1 Introduction

Pesticides contaminate groundwater and soil (Odukkathil and Vasudevan 2013; Verma et al. 2014) and negatively affect biodiversity (Geiger et al. 2010). Since the launch of Directive 91/414 in 1992, 74 % of the plant protection products have been removed from the European market or have been unapproved after review (EU-Commission 2009). While this process resulted in the withdrawal of most of the pesticides applied to the soil, alternative nontoxic solutions to these chemicals have yet to be developed. Additionally the pesticides remaining still pose a serious risk to the soil and environmental health. The lack of biopesticides for soil pathogen and pest control can be partially attributed to their inconsistent efficacy and to the poor characterization of their activity under biotic and/or abiotic stresses, relative to the more readily available chemical pesticides. Regarding abiotic stress, there is clear evidence that climate change is altering the distribution, incidence, and severity of diseases and pests. Elevated temperature increases pest developmental rate and number of generations per season (Somasekhard and Prasad 2012). Clearly there is an urgent need for developing reliable biorational and environmentally safe methods for the control of soil pathogens and pests. Here we review the use of compost, biofumigation, and bacterial biological control. Additionally we present results from Israel using biofumigation for pathogen and pest control and hypovirulent isolates for the control of Fusarium wilt. While these measures were first intended for sustainable and organic agriculture, we believe they will be adopted in the near future by the conventional sector.

16.2 Compost

Composting involves the controlled microbial degradation of organic material and is most commonly conducted aerobically. Besides heat, aerobic composting produces ammonia, carbon dioxide, and water, while anaerobic decomposition

produces CH_4, CO_2, and many intermediate organic compounds (Golueke 1972). Optimal microbial activity for substrate decomposition requires that the temperatures of compost piles be maintained below 60°C. Careful temperature control also allows for the survival and increase of pathogen antagonists in compost such as actinomycetes and *Bacillus* spp. (Hoitink and Fahy 1986). Composts can vary according to the initial raw material used, the chemical composition of the wastes (especially the lignin and cellulose content), the presence of beneficial microflora, and the conditions during the composting process (Hoitink and Boehm 1999; Noble and Coventry 2005). The disease-suppressive capability of composts is associated with microbial activity of bacterial and fungal populations in the rhizosphere and the interactions between antagonists and pathogens. Composts contribute to the suppressive activity of the amended soils through one or a combination of (a) competition for nutrients, (b) antibiosis, (c) production of lytic and other extracellular enzymes and compounds, (d) hyperparasitism and predation, and (e) host-mediated induction of resistance or abiotic factors such as pH and C-source (Hoitink et al. 1997; Whipps 1997; Steyaert et al. 2003; El-Masry et al. 2002; Keswani et al. 2014; Stoffella and Kahn 2001; Malandraki et al. 2008; Hoitink and Fahy 1986; Alfano et al. 2007; Yogev et al. 2010; Borrero et al. 2004; Bisen et al. 2015, 2016). As a result, it is often difficult to determine the exact suppression mechanisms, especially in compost due to the complex structure of the microbial community in compost (Boulter et al. 2000, 2002).

The potential of composts to suppress soilborne pathogens such as *Pythium* spp., *Rhizoctonia solani*, and *Fusarium* spp. has been demonstrated in many studies (Erhart et al. 1999; Hoitink et al. 1997; Borrero et al. 2006). Under normal production circumstances, when inoculum pressure is not drastic, or when inoculum is weakened (Freeman and Katan 1988), compost-derived pathogen suppression may serve as a practical control tool. Suppression of Fusarium diseases by composts was reported for a single combination of compost and pathosystem, viz., coffee-waste composts for controlling Fusarium wilt in melon, tomato residues to control Fusarium crown and root rot in tomato, and separated cattle manure for controlling Fusarium root and stem rot in cucumber and Fusarium crown and root rot in tomato (Ros et al. 2005; Cheuk et al. 2005; Kannangara et al. 2004; Raviv 2005). Yogev et al. (2006) demonstrated suppression of diseases caused by different formae speciales of *Fusarium oxysporum* by composts based on plant-waste residues. Composts derived from plant residues suppressed diseases caused by *Fusarium* pathogens, as compared with disease development in the highly conducive peat. The composting of plant residues not only provides suppressive composts but also has high sanitation value, since composting eliminates surviving pathogens from the infected tissues (Hoitink and Fahy 1986). Composting of plant residues is also of significant environmental value as it avoids the need for landfills (Yogev et al. 2006).

In addition to the suppressive effect of compost on plant pathogens, long-term compost amendments supported higher levels of mites belonging to the suborders Mesostigmata and Oribatida (Leroy et al. 2007; Minor and Norton 2004). Mites (Acari) of these taxa perform important ecological functions in the soil, serving as predators (mostly from Mesostigmata) and decomposers (Oribatida) (Manu and

Honciuc 2010; de Groot et al. 2016). Acarine soil predators feed on insects, mites, nematodes, and fungi (Gerson et al. 2003). One acarine soil pest of economic importance of flowers, vegetables, and cereals is the bulb mite *Rhizoglyphus robini* (Gerson et al. 1983, 1985; Diaz et al. 2000). Biological control of this pest in lily began with the identification and evaluation of the generalist predator *Gaeolaelaps aculeifer* (Lesna et al. 1995, 1996; Conijn et al. 1997; Lesna et al. 2000). Another avenue of research that was pursued was the association between *Fusarium oxysporum* and *R. robini*. Plant damage and mite populations were significantly higher when both pathogen and mites were present (Okabe and Amano 1991). Additionally, the mite was attracted to alcohols secreted by this fungus, on infested bulbs (Shinkaji et al. 1988; Okabe and Amano 1990). Recently it was demonstrated that *R. robini* is attracted to feed on plant tissue infested with *F. oxysporum* but will not attack healthy onion seedlings (Ofek et al. 2014; Lebiush-Mordechai et al. 2014). For future research testing, the hypothesis that onions and garlic grown in suppressive soils, antagonistic to *F. oxysporum*, created by applying mature compost will harbor lower levels of *R. robini* and have reduced plant damage (Yogev et al. 2006).

Another contributing factor to onion and garlic damage associated with *R. robini* and plant pathogens is the plant-parasitic nematode *Ditylenchus dipsaci*. Interestingly, compost amendments have been shown to reduce plant-parasitic nematodes while enhancing nonpathogenic, free-living nematodes (Thoden et al. 2011; Korthals et al. 2014). This in turn becomes relevant for the biocontrol of *R. robini*, other arthropod pests, and plant-parasitic nematodes because these free-living bacteriophagous nematodes are an excellent food source for generalist and specialized predatory mites (Heidemann et al. 2014; Britto et al. 2012; Read et al. 2006).

16.3 Biofumigation

Biofumigation is a cultural method to treat soilborne pests, usually as green manures, but also as crops in crop rotation, intercrops, and pure compound amendments as seed meal, dried plant material, or soil mulching (Tsror (Lahkim) et al. 2007; Davis et al. 1996; Muehlchen et al. 1990; Reddy 2013). Most commonly used for biofumigation are plant crops containing glucosinolates (GLS). GLS are sulfur-containing plant metabolites with basic skeleton consisting of a β-thioglucose residue, an N-hydroxyiminosulfate moiety, and a variable side chain (Fahey et al. 2001). Over 130 nitrogen- and sulfur-containing GLS compounds have been identified in vegetative and reproductive tissues of 16 plant families, mainly in the order Capparales, Brassicaceae, Capparaceae, Moringaceae, Tovariaceae, and Resedaceae (Brown and Morra 1997). GLSs are hydrolyzed by the myrosinase enzyme (present endogenously in *Brassica* tissues) to release a range of hydrolysis products including oxazolidinethiones, nitriles, thiocyanates, and various forms of volatile isothiocyanates (ITCs). These hydrolysis products, particularly ITCs, are known to have broad biocidal activity including insecticidal, nematicidal, fungicidal, antibiotic, and phytotoxic effects (Brown and Morra 1997; Kirkegaard and Sarwar 1998; Ploeg 2008; Reddy 2013). Biofumigation may affect the microflora composition and dynamics

of microorganism populations, resulting in competition for resources (Shetty et al. 2000; Smolinska et al. 1997; Smolinska and Horbowicz 1999). Other plants that have shown the potential to serve as biological fumigants are members of the Gramineae family producing a rich diversity of bioactive chemical compounds, including phenolics, glycosides, and a cyanogenic glucoside compound called dhurrin, that breaks down to release toxic cyanide when plant tissue is damaged (Yamane et al. 2010). Yet another group of bioactive plants are those in the Alliaceae family where the primary volatile compounds are thiosulfinates and zwiebelanes, mainly converted in soil or in *Allium* products (extracts) to disulfides (Block et al. 1992). Biofumigation technology demonstrates the potential to reduce environmental pollution through the replacement of toxic synthetic pesticides with biodegradable plant secondary metabolites. Crops grown with biofumigation could be marketed as pesticide-free, thereby enhancing the potential for increased profits for growers (Reddy 2013).

While there is considerable literature on the use of biofumigation on soil pathogens and nematodes as reviewed above, much less can be found on the control of soilborne insect pests. Here the emphasis will be on field rather than laboratory experiments. We also present studies on the compatibility of biofumigation with natural enemies.

16.3.1 Effects of GLS on Insect Pests

Different mechanisms have been proposed for GLS mode of action against pests. These include inactivating the thiol group of essential enzymes of the pest or alkylating the nucleophilic groups of biopolymers like DNA or as uncouplers (Tsao et al. 2002). Due to uncoupling between the respiratory chain and phosphorylation, respiration is accelerated, requiring more adenosine triphosphate (ATP) as an energy source, and at the same time, ATP production is blocked. This causes exhaustion of stored energy sources which finally leads to the death of the pest (Vig et al. 2009). The effect of GLS on an insect pest may vary depending on the type of ITCs produced, for example, the contact toxicities of methyl, propyl, allyl, phenyl, benzyl, and 2-phenylethyl ITCs were tested in the laboratory on eggs of the black vine weevil *Otiorhynchus sulcatus*. This weevil is a serious economic pest of high-value nursery, greenhouse, and field crops, where the larvae feed on the roots. All ITCs tested were toxic to the weevil eggs; however, ITCs containing an aromatic moiety were considerably more toxic than aliphatic (methyl, propyl, and allyl) ITCs. These results suggest that soil amendments of *Brassica* spp. tissues producing aromatic ITCs may have a greater insecticidal potential than those producing aliphatic ITCs (Borek et al. 1995). When *Brassica juncea or Sinapis alba* seed meals were evaluated in field experiments against *O. sulcatus*, *B. juncea* meal treatments resulted in 100 % weevil mortality, while *S. alba* meal had no effect on weevil mortality (Brown et al. 2004). Methyl-ITCs were also toxic to the soil-inhabiting white-fringed weevil larvae, *Naupactus leucoloma* (Matthiessen and Shackleton 2000). *Brassica juncea* seed meal incorporated into the top potting medium was also found efficient in

controlling the fly *Bradysia impatiens* (fungus gnats) (Diptera: Sciaridae), known as a serious pest in nurseries and greenhouses, as the females lay eggs in moist organic matter or potting media and the larvae feed on the plants (Main et al. 2014).

Wireworms, a widespread and important soilborne crop pest, can also be controlled with allyl isothiocyanate (AITC). Soil amended with allyl glucosinolate, present in relatively high concentrations in *Brassica oleracea, B. juncea*, *B. carinata*, and *B. nigra*, was found to have acute toxicity to the wireworm *Limonius californicus* (Williams et al. 1993). On the other hand, meal of *B. napus* amended (3 % on a weight basis) to soil repelled wireworms *L. californicus* but in uncovered containers did not kill them (Brown et al. 1991). Other species of wireworms, *Agriotes brevis*, *A. sordidus*, and *A. ustulatus*, were also affected by chopped fresh plants from *B. juncea* sel. ISCI 99 and biofumigant meals derived from defatted seeds of *B. carinata* sel. ISCI 7 in both laboratory and field trials. Defatted seed meals, applied at a rate to release 160 μ moles of glucosinolate L^{-1} of soil, caused very high larval mortality and prevented wireworms from damaging seedlings. The insecticidal effect of the chopped whole plants of *B. juncea* was less consistent (Furlan et al. 2010). Masked chafer beetle *Cyclocephala* spp. larvae is a common soil-inhabiting pest of ornamental plants, turfgrasses, corn, and soybean. Soil amended with *B. juncea* tissue was also effective against larvae of this beetle. AITC levels were positively correlated to larval mortality, with 8 % *B. juncea* treatment resulting in 100 % larval mortality with an average AITC concentration of 11.4 mg L^{-1} of soil (Noble et al. 2002). Indole-3-acetonitrile found in *B. oleracea* inhibited the growth of moths of the Pyralidae family, of the European corn borer *Pyrausta nubilalis*, and of the honeycomb moth *Galleria mellonella* (Smissman et al. 1961).

16.3.1.1 Effects of Sulfides on Insects

Damaged *Allium* plants produce and release sulfur allelochemicals, presumably to prevent insect herbivory. Defensive sulfur compounds, particularly dimethyl disulfide (DMDS), are highly toxic for nonadapted species. The toxicity of DMDS in these insects is due to the disruption of the cytochrome oxidase system in the mitochondria (Dugravot et al. 2004). The insecticidal activity of garlic essential oils was evaluated on the Japanese termite *Reticulitermes speratus*. The compound most toxic to the termite was diallyl trisulfide, followed by diallyl disulfide (Park and Shin 2005). Dimethyl disulfide (DMDS) and dipropyl disulfide (DPDS) led to the mortality of Coleopteran *Bruchidius atrolineatus* (Nammour et al. 1989). DMDS also killed the cowpea seed beetle *Callosobruchus maculatus*. This beetle develops during its postembryonic growth in the seeds of the cowpea *Vigna unguiculata* and causes high losses during storage of these seeds (Dugravot et al. 2004).

16.3.1.2 Effect of Biofumigation on Nontarget Arthropod Communities

Few studies have investigated the effects of biofumigation on nontarget organisms. The effects of mustard varieties used as early-season cover crops on cucumbers or pumpkins grown as the subsequent crops were investigated in Illinois, the USA, on

nontarget arthropod communities. Brown (Indian) mustard, *Brassica juncea* var "Florida broadleaf," and yellow (white) mustard *Sinapis alba* var "Tilney" were used. The abundance of ground beetles (Coleoptera: Carabidae), springtails (Hexapoda: Collembola), and mites (Arachnida: Acari) was monitored before, during, and after the seeding, growth, and incorporation of the two mustard varieties, individually or in combination, and in plots where no cover crops were grown. Overall, results indicated that mustard cover crops had minimal effects on the abundance, diversity, species richness, and overall community dynamics of the taxa investigated, suggesting it to be an environmentally safe method for the suppression of weeds and plant pathogens and for the increase of soil tilth (Dold 2010).

Similar results were attained in a study where the effect on soil organisms of four different white cabbage *B. oleracea* cultivars exhibiting a high degree of intraspecific variation in root glucosinolate profiles was compared. Intraspecific variation affected root-feeding nematodes, whereas decomposer organisms such as earthworms and Collembola were not affected. These results show that variation in root chemistry predominantly affects belowground herbivores and that these effects do not extend into the soil food web (Kabouw et al. 2010).

On the other hand, there are examples of negative effects of biofumigation on parasitoids. In West Africa the cow seed beetle *C. maculatus* causes major losses during the seed storage of the cowpea *V. unguiculata*. The larvae of *C. maculatus* are parasitized inside the seeds by *Dinarmus basalis*. African farmers introduce aromatic plants into storage systems at the beginning of the storage period that release toxic volatile compounds into the headspace of the stores. The susceptibility of *C. maculatus* and *D. basalis* to two plant sulfur-containing compounds, methyl isothiocyanate (MITC) and dimethyl disulfide (DMDS), was analyzed under laboratory conditions. The adults of *C. maculatus* and *D. basalis* had the same susceptibility to MITC, but the parasitoid was more susceptible to DMDS than its host. The higher susceptibility of *D. basalis* to the treatments could have consequences on biological control in storage systems. When *D. basalis* adults were put into the storage systems in the absence of *Boscia senegalensis* (Capparaceae) leaves, successive generations of the parasitoids maintained the *C. maculatus* population at a low density. However in the presence of *B. senegalensis* leaves, the *D. basalis* population was more affected by the treatment than its host *C. maculatus* and was thus incapable of preventing the increase in the pest population. This traditional method appears to enhance seed damage by limiting the efficiency of biocontrol (Dugravot et al. 2002).

16.4 Bacterial Biological Control of *Fusarium* spp.

Being a soil pathogen, *Fusarium oxysporum* is difficult to control, and Fusarium disease prevention is limited. Crop rotation is not a useful control method since the fungus can survive in the soil for long periods of time. Other methods include planting resistant plants, clean seeds, and systemic fungicides. In recent years, biocontrol

of *F. oxysporum* diseases has become an alternative to the chemical fungicides. Over 70 years ago, biological control of soilborne pathogens was proposed (Baker and Snyder 1965). Two approaches were perceived: firstly, enhancement of the naturally occurring populations of antagonists and, secondly, introduction of a selected biocontrol agent (BCA). Since then, the number of publications reporting positive control of soilborne diseases has increased dramatically, and several BCAs are commercially available including bacterial BCAs (BBCAs). Two groups of BBCAs for Fusarium wilt disease and Fusarium crown disease were investigated and developed: endophytic bacteria living within the plant and plant growth-promoting rhizobacteria (PGPRs) that colonize the rhizosphere; in this review we focus on the latter.

16.4.1 Endophytic Bacteria

Endophytic bacteria are known to enhance plant health in crops through various mechanisms: phosphate solubilization, nitrogen fixation, siderophore production, phytohormone and enzyme production, induction of host resistance, and biological control of plant pests and diseases (Ryan et al. 2008; Rosenblueth and Martínez-Romero 2006). Ho et al. (2015) used *Burkholderia cenocepacia* 869 T2, and Tan et al. (2015) used *Serratia marcescens* ITBB B5-1 for the control of banana Fusarium wilt. Six endophytic bacteria isolated from cotton, *Aureobacterium saperdae*, *Bacillus pumilus*, *Phyllobacterium rubiacearum*, *Pseudomonas putida*, *P. putida*, and *Burkholderia solanacearum*, reduced disease severity when inoculated to cotton seedlings (Chen et al. 1995).

16.4.2 PGPRs

PGPRs are beneficial rhizospheric bacteria that directly or indirectly affect plant growth and fitness (Saraf et al. 2014). The rhizobacteria *Pseudomonas fluorescens* was reported to synthesize antifungal antibiotics, such as 2,4-diacetylphloroglucinol, which inhibits the growth of phytopathogenic fungi (Nowak-Thompson et al. 1994), while other PGPR strains drastically reduced fusaric acid produced by *F. udum*, the causal agent of Fusarium wilt disease in pigeon pea (Dutta et al. 2008). *P. fluorescens* showed direct antagonistic activity against *F. oxysporum* found in rice and sugarcane by the production of antifungal metabolites (Kumar et al. 2002). *Pseudomonas stutzeri* produced extracellular chitinase and laminarinase which lysed the mycelia of *Fusarium solani* (Lim et al. 1991). Soil inoculation with phosphate-solubilizing bacteria managed the wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* (Khan et al. 2007). *Bacillus amyloliquefaciens* W2, isolated from the soil of *Crocus sativus* fields in India, successfully reduced the wilt disease rate of *Crocus sativus* in vitro and in a potted plant (Gupta and Vakhlu 2015). *Paenibacillus*

sp. 300 and *Streptomyces* sp. 385 suppressed Fusarium wilt of cucumber caused by *F. oxysporum* f. sp. *cucumerinum* in a nonsterile, soilless potting medium due to their chitinolytic activity (Singh et al. 1999).

In a study conducted by Omar et al. (2006), soil inoculation of BBCA combined with a fungicide showed synergistic activity. Two antagonistic bacteria, *Bacillus megaterium* C96 and *Burkholderia cepacia* C91, were examined for the control of *F. oxysporum* f. sp. *radices*, the causal agent of crown and root rot of tomato alone or combined with the fungicide carbendazim. The fungicide had almost no effect at concentrations lower than 50 μg ml^{-1}, and each bacterial isolate showed 20 % reduction in the disease symptoms. However, combining 1 μg ml^{-1} of carbendazim with *B. cepacia* C91 and 10 μg ml^{-1} carbendazim with *B. megaterium* C96 reduced disease symptoms by 46 % and 84 %, respectively. de Boer et al. (2003) showed synergistic activity using combinations of *P. putida* strains. When WCS358 and RE8 strains were mixed into the soil, disease suppression was significantly enhanced to approximately 50 % as compared to 30 % reduction for the single-strain treatments. The authors suggested that the synergistic effect was the result of the combination of different disease-suppressive mechanisms. The combination of the two chitinolytic bacteria *Paenibacillus* sp. 300 and *Streptomyces* sp. 385 provided better suppression of Fusarium wilt of cucumber than when each was applied separately (Singh et al. 1999). Similarly Park et al. (1988) reported improved suppression of Fusarium wilt on cucumber by the combination of *P. putida* and nonpathogenic isolates of *F. oxysporum*.

Formulated BBCAs usually provided better disease suppression than the unformulated BBCAs. Singh et al. (1999) showed that of several formulations tested for *Paenibacillus* sp. 300 and *Streptomyces* sp. 385, the zeolite-based chitosan-amended formulation provided the best protection against the Fusarium disease. El-Hassan and Gowen (2006) tested different formulations for BBCA *B. subtilis* used for protecting lentil against the wilt disease caused by *F. oxysporum* f. sp. *lentis*. Seed treatments with formulations of *B. subtilis* based on glucose, talc, and peat significantly enhanced biocontrol activity against Fusarium compared with a treatment in which spores were applied directly to seed. Bora et al. (2004) used talc-based seed treatment formulations of two strains of *P. putida* (strain 30 and strain 180) to suppress the development of Fusarium wilt of muskmelon, caused by *F. oxysporum* f. sp. *melonis*. In field trials, control efficacy of *P. putida* strains 30 and 180 were 63 % and 50 %, respectively, while the fungicide benomyl used as a commercial reference was less effective. Zacky and Ting (2015) evaluated sodium alginate, kaolin clay, and alginate-kaolin formulations for the BBCA *Streptomyces griseus*. Results indicated that formulated cells of *S. griseus*, irrespective of the materials used, were generally more effective in inhibiting growth of *F. oxysporum* than nonformulated cells.

To summarize BBCAs play an important role in preventing and suppressing plant diseases. BBCAs isolated from ecological niches, suitably formulated, have successfully reduced Fusarium disease in short-term experiments conducted in

potted plants, in greenhouses, and in the field. Further BBCAs research is needed to attain long-term effects on Fusarium disease reduction.

16.5 Biorational Control of Soil Pathogens and Pests in Israel

16.5.1 Biofumigation for the Control of Fusarim Wilt

The effects of growing *Brassica* as green manure crops preceding tomato production were investigated in two field trials (commercial-size greenhouses) in the B'sor region in the southwest of Israel. Green manure cover crops including two mustard *Brassica juncea* cvs. ("red giant" and "99"), arugula *Eruca sativa*, and broccoli *B. oleracea* were compared to fallow treatment. Fusarium wilt incidence in 2007 was reduced from 20.8 % in fallow treatment to 8.3–17.7 % in the green manure crops and, in 2008, from 19.4 % in fallow to 7.4–13.9 % in the green manure crops, but with no significant differences (Table 16.1). Yields were not statistically affected by the cover crops. These results are in accordance with Hartz et al. (2005), who also reported that mustard crops were ineffective in suppressing soilborne disease or improving tomato yield, apparently due to the high level of pathogen density.

16.5.2 Hypovirulent Isolates for the Control of Fusarium Wilt

Biocontrol agents have the potential to manage various soilborne diseases including Fusarium wilt, crown rot, and root rot of tomato. Among the antagonists that have shown satisfactory degrees of control against these diseases are *Trichoderma*

Table 16.1 Effect of green manure crops preceding tomatoes on Fusarium wilt incidence (90 days after planting) and yield

	2007		2008	
	Fusarium wilt (%)	Yield (kg/plot)*	Fusarium wilt (%)	Yield (kg/plot)**
Broccoli	11.5	43.1	13.9	1.39
Arugula	8.3	44.8	8.3	1.13
Mustard cv red giant	17.7	44.3	12	1.21
Mustard cv 99	10.4	44.9	7.4	1.1
Fallow	20.8	41.1	19.4	1.27

Trials were conducted in a randomized block design with four replications (24–27 plants/replicate)

Green manure crop sowed in October 2006, November 2007; incorporation of biomass into the soil: January 2007, February 2008; cherry tomato (cv. 139) planting dates: February 2007, April 2008

* 9 harvests; ** 2 harvests

Disease symptoms were analyzed by analysis of variance (ANOVA). Means were compared with student's multiple range test at a significance level of $P < 0.05$. Percentages were arcsine transformed before analysis

harzianum (Sivan et al. 1987), hypovirulent binucleate *Rhizoctonia* (HBNR) (Muslim et al. 2003), and nonpathogenic *Fusarium* species which decreased the population of the pathogenic *F. oxysporum* in roots and contributed to disease suppression. Nelson et al. (1992) also reported that pre-inoculation of tomato and cucumber with nonpathogenic *Fusarium* species reduced the frequency of isolation of pathogenic formae speciales. Biocontrol of Fusarium crown and root rot of tomato was obtained by cross protection and pre-inoculation of hypovirulent strains of *F. oxysporum* preceding inoculation with the virulent strains *F. oxysporum* f. sp. *radicis-lycopersici* (unpublished data) (Kortnizki-Shapira 1998). *Fusarium* strains (230) isolated from 21 sites (including agricultural fields with organically grown wheat, potato, carrot, celery, radish, cabbage, and tomato; nonagricultural open fields) were tested for pathogenicity to tomato seedlings. Among these isolates, 26 % classified as hypovirulent, with no disease symptoms or light symptoms (of brown lesions), were selected for cross-protection trials (data not shown). In cross-protection trials where hypovirulent *Fusarium* isolates were applied to tomato seedlings and a week later inoculated with a virulent *F. oxysporum* f. sp. *radices-lycopersici*, disease severity was significantly reduced by 25–86 % (Table 16.2). To conclude, hypovirulent isolates are potential candidates to promote the development of biocontrol agents and to reduce the use of chemicals for disease control.

16.5.3 Biofumigation for Mealybug Control on Fresh Herbs

Mealybugs are considered serious pests of various crops feeding on plant sap on roots and foliage (Kaydan et al. 2015). In an experiment conducted in 2010 in northern Israel, researchers evaluated the effect of biofumigation with three Brassicaceae species, *B. oleracea*, *B. napus*, and *Eruca sativa*, with fresh and dried plants compared to an untreated control, in two cropping systems: *Planococcus citri* on *Mentha spicata* and *Phenacoccus solani* on *Artemisia dracunculus*.

The experiments were conducted in a 400 m^2 screenhouse, and the herbs were grown in 10 L flowerpots in local basalt soil, mixed with the respective amendments described below. Brassicaceae plants used as dry amendments were harvested and dried in the shade to 20 % of their fresh weight and applied at 0.2 % of soil weight. The fresh amendments were harvested just before incorporating into the soil and applied at 1 % of soil weight. The herbs were planted on two dates, on the day the amendments were added to the soil and a month later, replicated ten times per treatment in both systems. Each plant was inoculated with ten adult mealybugs. Mealybug populations (adults and nymphs) were monitored on each plant every week for 2 months until harvest. At harvest each plant was uprooted and all the mealybugs from root to shoot were counted and the condition of each plant was rated from zero (dead plants) to five (foliage in excellent condition).

Biofumigation treatments had a negative effect on the establishment and development of the mealybugs in the two systems. On *A. dracunculus* the effect on

Table 16.2 Effect of hypovirulent *Fusarium* strains application to tomato prior to inoculation with *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) on disease symptoms

Isolate	Site/host	Symptoms on tomato plants [x]	% disease reduction [y]
Trial 1			
FORL II*	Tomato	2.71 a [z]	–
FORL I**	Tomato	2.62 a	–
AZ	Noncultivated field	1.51 b	44
BE-10	Nursery	0.86 c	68
NI-2	Garlic field	0.80 c	71
KI-32	Radish field	0.80 c	71
NZ-3	Netzer	0.74 c	73
TY-5	Celery field	0.70 c	74
NZ-11	Netzer	0.69 c	75
TY-19	Celery field	0.66 c	76
G-11	Hatzeva	0.65 c	76
B-10	Unknown	0.62 c	77
BE-35	Nursery	0.60 c	78
NO-18	Carrot field	0.58 c	79
Check***		0.45 c	86
Trial 2			
FORL II*	Tomato	2.50 b	–
FORL I**	Tomato	2.35 b	–
TY-18	Celery field	1.89 c	25
NT-21	Onion field	1.47 cd	41
KI-29	Radish field	1.14 d	55
NT-11	Corn field	0.71 e	72
NZ-8	Netzer	0.69 e	73
SU-4	Ein-Yahav	0.68 e	73
B-4	Unknown	0.59 e	73
JE-6	Tzomet	0.48 ef	81
MO-10	Hatzeva	0 f	100
Check***		0.28 ef	86

Two experiments in a completely randomized design were conducted in climate chambers. In each treatment 45–60 plants. Conidial suspension of hypovirulent *Fusarium* isolates was applied to tomato seedlings (cv. M-82; 14-days old), and inoculation with a virulent *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) was performed a week later

* Inoculated with the virulent FORL 7 days after hypovirulent *Fusarium* application

** Inoculated with the virulent FORL at time of hypovirulent *Fusarium* application

*** Non-inoculated control

[x]Disease symptoms were determined 10 weeks after inoculation with FORL on a scale of 0–5, where 0 = no symptoms, 1 = low infection with lesions smaller than 1 mm diameter, 2 = moderate infection with lesions size above 1 mm, 3 = high infection with large lesions, 4 = full girdling at stem base, and 5 = death of plant

[y]Reduction in plant symptoms as compared with FORL II

[z]Disease symptoms were analyzed by analysis of variance (ANOVA). Means were compared with student's multiple range test at a significance level of $P < 0.05$. Different letters within a column indicate a significant difference

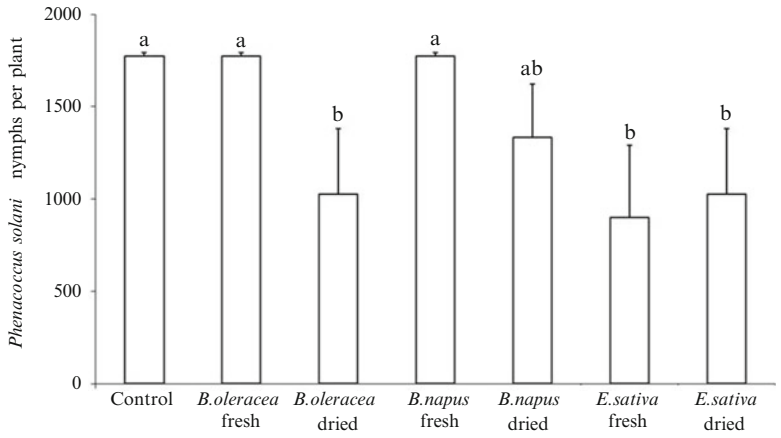

Fig. 16.1 Total numbers of *Phenacoccus solani* nymphs (average ± S.E.) on tarragon *Artemisia dracunculus* plants at harvest in different biofumigation treatments incorporated a month before the herbs were planted

Note: Columns marked with different letters differ significantly at $p < 0.05$ (Tukey-Kramer HSD means comparison test)

P. solani lasted for 2 months until harvest (Fig. 16.1), while on *M. spicata*, the effect on *P. citri* lasted only 1 month (Fig. 16.2), and at harvest there was no difference in *P. citri* adult population size between the treatments ($F_{6.63} = 0.8$; $p = 0.59$).

The effect of biofumigation with canola in the *A. dracunculus* system on *P. citri* was not significantly different from the control (Fig. 16.1), while in the *M. spicata* system, it was as good as *B. oleracea* and *E. sativa* (Fig. 16.2).

Drying *B. oleracea* before application to the soil improved its ability to control *P. solani* (Fig. 16.1), while in the other Brassicaceae, the condition at application did not affect the results (Figs. 16.1 and 16.2). In addition, differences were found in herb plant response: *M. spicata* plants benefited from the biofumigation at the beginning compared to control (2 weeks after planting chi-square = 14.92; $p = 0.02$), and at harvest there was no difference between the plant condition in the different treatments (chi-square = 3.26; $p = 0.78$). On the other hand, there was 30 % mortality of the *A. dracunculus* plants in the fresh cabbage treatment and 20 % in the dry *B. oleracea* treatment.

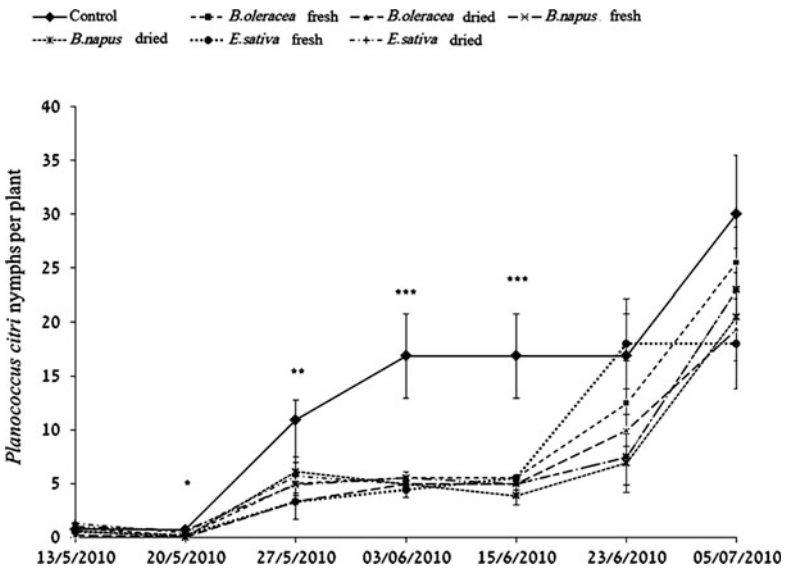

Fig. 16.2 Total numbers of *Phenacoccus citri* nymphs (average ± S.E.) on mint *Mentha spicata* plants in different biofumigation treatments incorporated on the same day the herbs were planted *Note: Dates with one, two, or three asterisks indicate a significant difference between treatments and the non-treated control at p < 0.05, <0.01, <0.001, respectively (one-way ANOVA)*

16.5.4 Conclusion

Our results along with the cited literature demonstrate the potential of biofumigation for the control of soilborne insect pests. It is important to consider that the efficacy of the same biofumigant plant on similar pests in different planting systems can vary. Additionally the phytotoxicity of the biofumigant plant on the crop and its compatibility to natural enemies need to be determined before implementing this technology in an integrated pest management (IPM) regime.

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Present Status and the Future Prospects of Microbial Biopesticides in Iran

17

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Abstract

A considerable part of agricultural products are destroyed in Iran by various pests such as insects, weeds, fungi, nematodes, viruses, and bacteria. Application of chemical pesticides has improved agricultural productivity, but they have also caused adverse effects on the environment and human health. Microbial pesticides (biopesticides) are often considered as low-risk substitute for conventional chemical pesticides that can play a key role in resolving the problem in a sustainable way. Though reducing agrochemical usage is a public demand in Iran, the adoption of biopesticides by growers has been much slower than expected. However, growing social awareness about the advantages of biocontrol promises bright prospects for the business of microbial pesticides in Iran. In this chapter we reviewed the history of biocontrol of pest in Iran and its future prospects. We also discussed the issues that restrain the interest of Iranian companies to fund research and development of biopesticides and hinder their acceptance by Iranian growers. Available biopesticides and the list of producing/distributing companies in Iran are also included.

Keywords

Biopesticide • Agrochemicals • Biocontrol • Pest

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17.1 Introduction

Iran is placed in West Asia and is adjacent to the Caspian Sea, Persian Gulf, and Gulf of Oman. It is the 18th largest country of the world with an area of 1,633,189 square kilometers (Kiani Haftlanf 2003), with only one-third of its total surface area suitable for farming. However, water deficiency in recent years reduces its cultivable land area (arable land, orchards, and vineyards) to only 10.4 % (FAO Stat 2015a).

Many insects, weeds, and pathogens (such as fungi, nematodes, viruses, bacteria, and so on) adversely affect agriculture in Iran, either by yield loss or quality loss. Application of chemical pesticides has been a routine management method for controlling these pests; however, their hostile impact on the environment and human life is undeniable (Moosavi and Zare 2012). The use of chemical pesticides has increased tremendously in the last few decades because of the need for more food from the diminishing natural resources.

Although the application of pesticides has improved agricultural productivity, they have also exhibited unfavorable effect on human, soil, and water health as well as developing crises such as pest resistance, pest resurgence, outbreak of secondary pests, poisonous residues in food or feed, and genetic variation in plants (Al-Zaidi et al. 2011). Human chronic diseases (such as cancers; diabetes; neurodegenerative disorders like Parkinson, Alzheimer, and amyotrophic lateral sclerosis; birth defects; and reproductive disorders) have increased abnormally in Iran, and there are many evidences that correlate with the rapid increase in incidence of these diseases with exposure to pesticides (Mousavi et al. 2009; Mostafalou and Abdollahi 2013). There is an urgent need to lessen the use of these damaging compounds by finding some alternative methods like biocontrol of pests (Vincent et al. 2007; Helyer et al. 2014). Application of biopesticides can play a key role in resolving the problem in a sustainable way. They provide a significant potential for implementation in sustainable agriculture by simultaneously satisfying the consumers, farmers, and industries.

The main focus of this chapter is to review the current status and prospects of research, development, and use of microbial biopesticides in Iran and address the issues that limit the production and implementation of these products in routine agriculture.

17.2 Iran's Agricultural Sectors

Though yearly precipitation averages 250 mm or less in most parts of the country, Iran is one of the few countries in the world that have all four distinguished seasons. About 64.9 % of the total area of Iran cannot be used for agricultural purposes because of deserts, salt flats and bare-rock mountains, cities, towns, villages, industrial areas, and roads. An additional 6.6 % is covered by forest and 18.1 % of the total area is exploited for grazing and small fodder production. The remaining cultivable land area is composed of 9.3 % arable land and 1.1 % for permanent crops (FAO Stat 2015a). In spite of the large number of workforce employed in agricultural activities, the farmlands are hardly economically viable in consequence of

Table 17.1 List of the most common products in Iran with their world ranking

World ranking[1]	Commodity
1	Berries, stone fruit, pistachios
2	Apricots, dates, walnuts, watermelons
3	Cherries, cucumbers and gherkins, eggplants (aubergines), melons (inc. cantaloupes)
4	Pumpkins, squash, and gourds
5	Almond, anise, badian, fennel, coriander, figs, quinces
6	Sour cherries, hazelnuts, lettuce, chicory, plums and sloes, tomatoes
7	Chick peas, peaches and nectarines, spinach, tangerines, mandarins, clementines, satsumas, tea
8	Kiwi fruit
9	Apple, lemons, and limes
10	Asparagus, grapes, persimmons
11	Lentils
13	Oranges, safflower seed
14	Carrots and turnips
15	Cabbages and other brassicas, citrus fruit, potatoes, sugar beet
16	Garlic, wheat
17	Beans
19	Grapefruit (inc. pomelos), pears, rapeseed

[1]Ranking is ordered by quantity of production in 2013

small size of the farms (less than 10 h), poor soil, water deficiency, low seed quality, and antiquated farming techniques. According to the latest FAO ranking for the largest global producer rankings (FAO Stat 2015b), the most important crops in Iran and their world production ranking are shown in Table 17.1.

The farmers try to increase their yield by any available measures, and one of the simplest ways is to intensify the agrochemical inputs. Only in the year 2013, more than 11,000 tons of active ingredients of pesticides (5261.8 tons of active ingredients of insecticides, 4144.77 tons of active ingredients of herbicides, and 1737.78 tons of active ingredient of fungicides and bactericides) were applied to agricultural products of Iran (FAO stat 2015a). These chemicals can produce many problems such as health concerns, unsafe food supplies, and environmental degradation (Partap 2010). Though reducing agrochemical usage is extensively appreciated by consumers as well as extension workers and researchers (Malek-Saeidi et al. 2012), the adoption of organic agriculture by growers has been much slower than expected. Area under organic agriculture in Iran was 11,601 hectares in 2014 that was equal to 0.02 % of the total arable land (Willer and Lernoud 2016). About 95 % of organic produce in Iran was exported in 2011 to European countries due to their high price for internal market (Kledal et al. 2012).

Biocontrol in Iran can be divided into three eras. It was initiated in 1931 by importing several vedalia beetles (*Rodolia cardinalis*) for control of cottony cushion scale (*Icerya purchasi*) on citrus orchards. Application of mealybug ladybird

(*Cryptolaemus montrouzieri*) against mealybugs (*Planococcus citri*) and a hymenopterous parasite (*Prospaltella berlesei*) against mulberry scale (*Pseudaulacaspis pentagona*) are the next two successful examples of biocontrol of pests in this era. The second era of biocontrol started from 1984 to 1995. During these years, biocontrol department was established at the "Iranian Research Institute of Plant Protection" where different disciplines were shaped. Collection, mass rearing, and application of *Trichogramma* wasps on various crops were carried out during these years.

Optimal Utilization of Fertilizers and Pesticides (OUFP) was the first of ten Iranian National Plans and the third era of biocontrol aimed to promote sustainable agriculture and decrease poisonous residuals in agricultural products. The program was started in 1995 but could not reach its target mainly due to low participation of growers (Sharifi-Moghaddam 2006). It seems that organic agriculture in Iran is still in its early phase (Soltani et al. 2014). Several pilot farms had been selected throughout Iran to promote organic agriculture and to demonstrate the effectiveness of biopesticides. The program known as Integrated Pest Management/Farmer Field School (IPM/FFS) has tried to attract more contributors for the dissemination of organic agriculture (Razzaghi-borkhani et al. 2010; Etehadi et al. 2011). The pilot farms included crops such as rice, cotton, corn, tomato, soybean, sugarcane, pea, citrus, tea, mulberry, pomegranate, pistachio, apple, and vine whose pests were controlled by seven biocontrol agents (*Trichogramma* wasps, *Bracon* wasp, green lacewing, mealybug ladybird, vedalia beetle, *Encarsia* (*Prospaltella*) wasp, and *Platytelenomus* wasp).

In spite of IPM/FFS enterprises, the Iranian growers have not adopted biocontrol at an acceptable level mainly because of challenges in certification, access to robust biocontrol agents, reliable technical information, marketing, and credits (Osku et al. 2007; Veisi et al. 2010; Rezvanfar et al. 2011; Karimi 2011; Moumeni-Helali and Ahmadpour 2013; Soltani et al. 2014).

17.3 Limiting Factors of Microbial Biopesticides in Iran

Biopesticides are often considered as reduced or low-risk substitute for conventional chemical pesticides. No resistance has been reported against biopesticides and they have little or no toxic residue effect on nontarget organisms. Notwithstanding the increasing interest in microbial biopesticides, their application has been faced with some difficulties that hamper their acceptance by Iranian growers. As well, several factors are involved in decreasing the interest of Iranian companies to fund research on microbial biopesticides. Some of the issues that restrain the production and use of these products are discussed in detail here.

17.3.1 Factors Limiting the Application of Microbial Biopesticides

The lack of complete profile of biopesticides is a main reason that microbial pesticides have not yet been considered as alternative to agrochemicals. The lack of

society awareness, distrust in farmers, and weakness of the supporting policy network are the main causatives for this flaw (Mishra et al. 2015; Kumar and Singh 2015; Keswani et al. 2013). However, it must be known that biological control alone will seldom provide complete control and it should be used in an integrated system with other measures. Raising the profile of microbial biopesticides among the community and policy makers may help in promoting their adoption by the farmers.

17.3.1.1 Intrinsic Problems of Microbial Biopesticides

Farmers only trust the products that are reliable, cost-effective, and user friendly (Marrone 2009; Regnault-Roger 2012; Moosavi and Zare 2012). One of the major problems that hinder the adoption of microbial biopesticides is inconsistency in their efficacy. Poor quality, quick decline of microbial propagules after introduction into soil in contest with soil microbiota, and effect of abiotic stresses are the main causes of inconsistency. Therefore, introducing the required population of the biocontrol agents is essential for successful control (Moosavi and Askary 2015). Farmers' trust can be increased when the commercial biocontrol agents are formulated as efficient, competent, competitive, and stress-tolerant products.

Poor shelf life (less than 1 year), need for special storage devices (that most sellers or farmers do not possess), and failure in mixing with conventional products (Moosavi and Zare 2015) are other limiting factors for acceptance of microbial biopesticides by Iranian growers (Cumagun and Moosavi 2015).

Pesticides containing biocontrol agents need more awareness of farmers. Conventional farmers with no or low knowledge and skills of application of biocontrol can hardly adopt these products. Training the farmers in developing countries (including Iran) for the concepts of biocontrol, advantages of microbial biopesticides, and destructive impact of chemicals is supporting inculcating acquaintance in the farmers' mind.

17.3.1.2 Limited Technology Dissemination and Promotion Programs

As mentioned before, several pilot farmlands have been selected in different parts of Iran in a program known as Integrated Pest Management/Farmer Field School (IPM/FFS) to exhibit the effectiveness of biopesticides in integrated pest management practices and to attract more participants for the dissemination of knowledge of organic agriculture (Razzaghi-borkhani et al. 2010; Etehadi et al. 2011). It is visible that the number of selected farms is not sufficient to effectively draw the farmer's attention to adopt biopesticides. On the other side, the most common measure in IPM/FFS has been the application of parasitoids and not microbial pesticides. Using high-quality microbial products in pilot farms can demonstrate the potency of biopesticides for the management of plant diseases, insect pests, and weeds. However, it must be explained to the growers that the effect of biopesticides is slower as compared to chemical pesticides. Biocontrol agents do not provide a rapid kill but rather suppress the pathogens or insect pests and manage them over time.

17.3.1.3 Low Clarity on the Microbial Biopesticide Residues in Food, Soil, and Water

In spite of several advantages of biopesticides, the information on their incidence and fate in food and environment is relatively low. This information is necessary for the protection of mankind, animals, and environment. Such data is also useful for regulatory purposes.

The European Union (EU) and the United States Environmental Protection Agency (USEPA) have set up "maximum residue limits" to define the highest amount of residue lawfully allowed in food (Munjanja et al. 2015). The usage of biopesticides may also result in the accumulation of residues in soil and water (Romero-González et al. 2015; Sanganyado et al. 2015). The information must be analyzed, adapted, and updated in Iran to establish as statutory law by which the consumers' and environment's safety is ensured. The maximum residue limits can be used as standards for universal trading.

Adversely, a part of Iranian society does not believe in microbial biopesticides and has no interest in having microbes in their food. Mass media can play a key role in training the society to avoid such a food phobia. Training of the distribution workforces, farmers, and society will enhance acceptance of microbial biopesticides.

17.3.2 Factors That Limit the Production of Microbial Biopesticides

17.3.2.1 Regulation and Registration

Only a registered biopesticide can be sold in the market. Production and appraisal of a complete registration procedure for a biopesticide is a very complicated, time-consuming, and expensive process. The outlay for safety trials and registration of a biopesticide is estimated to range between US$ 0.5 and 3 million over the time period of 4–8 years (Ehlers 2010; Ravensberg 2011; Bailey et al. 2011; Nollet 2016). Many companies cannot afford this amount of investment, especially small and medium companies. The situation will be worse if registration is required in each country (Whipps and Davies 2000).

Biopesticides are considered under the regulatory system which was established to deal with chemical pesticides. Obviously some aspects of the conventional system did not apply to biopesticides, and there is an urgent need to adjust the regulatory policy to facilitate their registration. Otherwise, the development and commercialization of biopesticides will be hindered.

There is also a risk for companies when the active ingredient (biocontrol agent) of the product can be easily isolated, specified, or used. Therefore, a strong intellectual property right (product or technological idea) is essential to protect the rights of active companies in the biopesticide sector (Tormala 1995; Moosavi and Zare 2015). Biological procedures, techniques, and organisms can be patented by spending; however, its power is debatable and the complexity will be increased by genetic engineering (Ravensberg 2011).

Government patronage is also vital for microbial biopesticide usage. The Iranian policy makers have started to modify the regulation to ease the process and decrease the registration costs. As well, new rules such as banning the excessive application of chemical pesticides and preventing trade of the products containing chemical residues more than specified are needed to encourage the use of biopesticides.

Development of a novel formulation of biopesticide requires a lot of money and time. It means that a corporation needs to invest millions of dollars which can only be sold after the product is registered. The market usually accepts the new products slowly and 5–8 years is required to reach to a break-even point. Lack of quick profit makes the venture unattractive to many investors. Government support will be of help to persuade investment in this sector.

The registration directive for biopesticide products is available on the Iranian Plant Protection Organization's website. The directive was adapted from FAO and USEPA guidelines for registration of biocontrol agents and updated according to quality control and mass production standards of biocontrol agents that were provided by the Biological Control Department, Iranian Research Institute of Plant Protection. The result was published on December 29, 2004, as Directive 2339/220/25 and came into effect on April 23, 2007, after final modifications made by faculty members of Iranian universities and the Iranian Research Institute of Plant Protection.

17.3.2.2 Market

The market share of biopesticides in the global crop protection market was 5 % (US$ 3 billion) in 2013. Biopesticide sales have grown with a compound annual growth rate of 8.64 %, and this is expected to continue increasing at the same rate until 2023, where the market will reach more than 7 % (US$ 4.5 billion) of the total global pesticide market (Olson 2015).

The market share of different biocontrol agents is not similar. It was estimated that about 74 % of the total biopesticide market was occupied by bacteria-based products and the remaining was occupied by fungal (10 %), predator (8 %), viral (5 %), and other (3 %) biopesticides (Thakore 2006). About 357 microbial biopesticides (105 in Australia, 101 in the USA, 39 in Europe, 33 in India, 30 in Africa, 21 in China, 19 in Iran, and 11 in Japan that include multiple registrations in different countries) were registered and sold all around the world (Mishra et al. 2015).

While the USA, Canada, and Mexico used approximately 45 % of the biopesticides, Asia only used 5 % of biopesticides sold throughout the world (Bailey et al. 2010). The biopesticide market's growth will partly come from gaining the market share of chemical pesticides and partly from new biopesticides that manage the pests which are not controlled with chemicals.

No official data on Iran's biopesticide market was available to be analyzed; however, its status is deducible from other information. Area under organic agriculture was exponentially decreased from 2011 (43,332 ha) to 2014 (11,061 ha) (Willer and Lernoud 2016). It was clear that higher price of organic products was deterrent to consumers. Limited domestic market and difficulty in exporting agricultural products may be other reasons.

However, like other countries the future of the business is very bright. The growing belief that correlates the chronic human diseases with chemical pesticides makes the consumers to seek for safer products with lower chemical residues. Though many people are now willingly ready to pay more money for safer food, the domestic market is still small. Government and media patronage is essential to orient the food demands to safer products. However, government subsidy can be of help in promoting the investment of companies on development of biopesticides and in decreasing the final price of organic products. Government support will assist the small businesses to survive in the market and supply biopesticides to niche markets.

17.4 Biopesticides Available in Iran

The largest proportion of the Iranian microbial biopesticide market is occupied by *Bacillus thuringiensis*-based products. *B. thuringiensis* was initially registered in Iran about 50 years ago (under the trade name of MVP® and Bactospin® on December 22, 1968, and May 9, 1975). The Bt products have been widely sold and used in different parts of Iran since then. Other microbial products in the market comprise mainly of fungal-based products (Table 17.2).

In spite of late registration of biopesticide, the succeeding growth in registration and application of these products were rather low. Lacking deterrent laws for the use of pollutant chemical pesticides, large companies preferred not to endanger their guaranteed profit with being involved with new production lines.

The existing biopesticides available for use in the Iranian market are both locally manufactured and imported. In the last decade, some small- and medium-scale companies have showed interest in this sector, but most of them have been still uncertain to invest in the venture because of the niche market and expensive registration process. The request and pressure from the NGOs or society as well as the assistance from the government have encouraged some other companies to overcome their hesitation and start the business. Four companies have so far been registered for producing biopesticides, and several products have been introduced to the market due to their efforts.

Many researchers have been working on biocontrol agents against different plant pests, and the results of their study are expected to translate in the Iranian market (Moosavi et al. 2010, 2011, 2015; Kalantari et al. 2013; Naraghi et al. 2014; Khorramvatan et al. 2014; Damani Zamani et al. 2015; Goudarzi et al. 2015; Salehi Jouzani et al. 2015; Gifani et al. 2015). In spite of slow and difficult development of the biopesticide market, undoubtedly enormous potential and opportunities exist in Iran that deserves consideration.

Table 17.2 List of microbial biopesticides and their producer/distributor companies in Iran

Company name	Product name	Active ingredient	Use against
Gyah Corporation	Mycotal	*Lecanicillium lecanii*	White fly and thrips larvae
	Trianum-P	*Trichoderma harzianum* strain T 22	Soilborne fungi
Kara Industrial Biotechnology Co.	BasioKara	*Beauveria bassiana*	Heteroptera and Coleoptera
	TrichoKara	*Trichoderma harzianum*	Plant pathogenic fungi
	MetaKara	*Metarhizium anisopliae*	Wide range of insect pests
Afrasam Co.	Naturalis	*Beauveria bassiana*	Sucking pests
	XenTari and FlorBac	*B. thuringiensis* subsp. *aizawai*	Armyworms and diamondback moth larvae
	BioBit and DiPel	*B. thuringiensis* subsp. *kurstaki*	Lepidoptera larvae
Biorun	BioBeet	*Bacillus thuringiensis* subsp. *san Diego*	Coleoptera larvae
	BioLep	*B. thuringiensis* subsp. *kurstaki*	Lepidoptera larvae
MehrAsia Biotechnology Company	Bithurin	*B. thuringiensis* subsp. *kurstaki*	Lepidoptera larvae
	Bithiran	*B. thuringiensis* subsp. *morrison*	Coleoptera larvae
	Biosubtil	*Bacillus subtilis*	Plant pathogenic fungi
Greenlife Biotech Co.	Tricomix HV	*Trichoderma harzianum*	Plant pathogenic fungi
	Orocide	*Fusarium oxysporum*	Broomrape
Foroughe Dasht Co.	RootGard	*Paecilomyces lilacinus*	Plant pathogenic nematodes
	BotaniGard	*Beauveria bassiana*	Wide range of insect pest

17.5 Conclusion

Growing social awareness about the hazardous effect of chemical pesticides on human and environment health, as well as the advantages of biocontrol, are the motivation forces in acceptance of biopesticides. Success of biopesticide products highly depends on government policies as it can affect the market size and promote the application of these products. The government can uphold biopesticide industries by enforcing laws banning hazardous agrochemicals and assigning subsidies and price support mechanisms and facilitate the export rules of organic agricultural products. The IPM/FFS program must be accomplished in more extensive areas to

get more farmers acquainted, especially those who work with traditional farming systems.

The active chemical industries are well equipped to launch novel products in the market, but the small- and medium-scale companies must be trained in this venture. They must learn how to invest on which kind of biocontrol agent, how to estimate the market size, and the hurdles in commercialization processes. A biocontrol agent will be successful if it has an extensive host range and high efficiency, easily inhabits the soil and compote with soil microbiota, and manages important pests in principle crops. However, complete success will be achieved if a potent biocontrol agent can emulate the traits of conventional chemicals.

Establishing powerful international linkage to receive academic, promotional, technical, and developmental support from international agricultural research and related institutes can help to increase the knowledge of biocontrol of pests. Several potent and indigenous biocontrol agents have been isolated from Iran that can potentially act as biopesticide. However, insufficient knowledge on formulation and commercial process hinder their introduction to market. There is an urgent need to bridge the gaps between research studies, commercialization, and application of microbial biopesticides.

Finally, in spite of bright prospects of biopesticide business in Iran, it should be considered that successful adoption relies on the complete cooperation of all participants (society, farmers, NGOs, government authorities, pesticide companies, stakeholders, retailers, and distributors).

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